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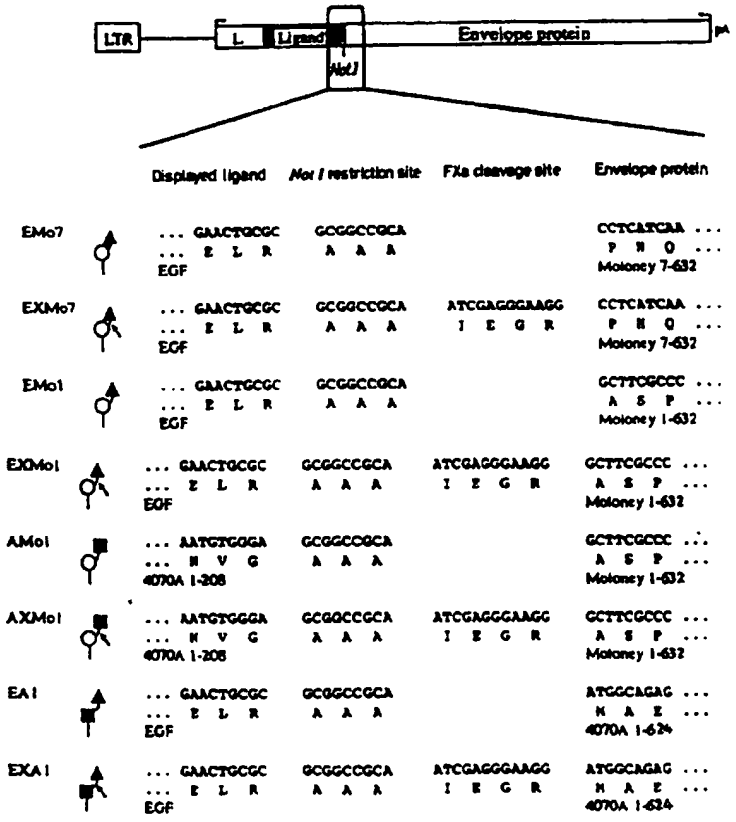
(54) Title: RECOMBINANT VIRUSES INCORPORATING A PROTEASE CLEAVABLE PROTEIN

(57) Abstract

Disclosed is a recombinant viral particle capable of infecting a eukaryotic cell, the viral particle comprising: a substantially intact viral glycoprotein fused, via an intervening linker region, to a heterologous polypeptide displayed on the surface of the particle, which heterologous polypeptide modulates the ability of the viral particle to infect one or more eukaryotic cell types and is cleavable from the viral glycoprotein by a protease acting selectively on a specific protease cleavage site present in the linker region, such that cleavage of the heterologous polypeptide from the viral glycoprotein allows the glycoprotein to interact normally with its cognate receptor on the surface of a target cell.



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Title: Recombinant Viruses Incorporating a Protease Cleavable Protein

Field of the Invention

The invention relates to recombinant viral particles incorporating protease cleavable proteins and to various applications of the recombinant particles.

Retroviral envelopes

Retroviral envelope glycoproteins mediate specific viral attachment to cell surface receptors and subsequently trigger fusion between the viral envelope and the target cell membrane. All retroviral envelope spike glycoproteins examined to date are homooligomers containing two to four heterodimeric subunits (Doms *et al.* 1993 Virology 193, 545). Each subunit comprises a large extraviral glycoprotein moiety (SU) noncovalently attached at its C-terminus to a smaller transmembrane polypeptide (TM) that anchors the complex in the viral membrane. In the case of murine C-type retroviral vectors, SU comprises two domains connected by a proline-rich hinge, the N-terminal domain conferring receptor specificity and exhibiting a high degree of conservation between murine leukemia viruses (MLVs) with different host ranges (Battini *et al.* 1992 J. Virol 66, 1468-1475). Moloney MLV envelopes confer an ecotropic host range because they attach selectively to a peptide loop in the murine cationic amino acid transporter (CAT-1), found only on cells of mouse and rat origin (Albritton *et al.* 1989 Cell 57, 659-666). 4070A MLV envelopes attach to an epitope on the ubiquitous RAM-1 phosphate symporter that is conserved throughout many mammalian species, and confer an amphotropic host range (Miller *et al.* PNAS 91, 78-82; VanZeijl *et al.* 1994 PNAS 91, 1168-1172). Thus, retroviral vectors with 4070A envelopes infect human cells promiscuously, whereas vectors with Moloney envelopes fail to infect human cells.

Proteolytic activation

In all retroviruses that have been studied to date, the SU and TM polypeptides are derived from a single chain precursor glycoprotein that undergoes proteolytic maturation in the

Gogi compartment during its transport to the cell surface. Uncleaved envelope precursor glycoproteins can be incorporated into viruses but are unable to trigger membrane fusion. The requirement for proteolytic maturation/activation is a feature common to the fusogenic membrane glycoproteins of many virus families and is most commonly mediated by the ubiquitous Golgi compartment serine protease, furin. However, there are well-documented examples of viral membrane glycoproteins that resist cleavage by ubiquitous intracellular proteases and instead are cleaved by secreted proteases available only in a few host systems (Klenk & Garten 1994 Trends Microbiol. 2, 39). Moreover, there is at least one example of an influenza virus strain whose haemagglutinin is activated by a target cell protease at the stage of virus entry (Boycott *et al.* 1994 Virology 203, 313). In these instances, the binding reactions of the viral membrane glycoproteins are unaffected by their proteolytic cleavage - only their ability to trigger membrane fusion is affected.

Retroviral display of nonviral polypeptides as N-terminal extensions of SU

A general method has been disclosed which allows the display of a (glyco)polypeptide on the surface of a retroviral vector as a genetically encoded extension of the SU glycoprotein (WO 94/06920, Medical Research Council). The polypeptide is fused (by genetic engineering) to the N-terminal part of the SU glycoprotein such that the envelope protein to which it has been grafted remains substantially intact and the fused nonviral polypeptide ligand is displayed on the viral surface. To date, the approach has been used to display many different polypeptide ligands on MLV - based retroviral vectors, including single chain antibodies, cellular growth factors and immunoglobulin binding domains (WO 94/06920, Medical Research Council; WO 96/00294 Medical Research Council; Cosset *et al.* 1994 Gene Therapy 1, S1; and Nilson *et al.* 1994 Gene Therapy 1, S17). In contrast to other chimaeric retroviral envelope proteins that have been described (CD4 chimaera, Kasahara *et al.* 1994 Science 266, 1373; Chu & Dornburg 1995 J. Virol. 69, 2659; Somia *et al.* 1995 PNAS 92, 7570) viral incorporation of N-terminally extended SU glycoproteins does not require the presence of unmodified envelope glycoproteins.

In principle, a virus displaying such a chimaeric envelope protein might be capable of multivalent attachment both to the natural virus receptor (via the N-terminal domain of SU) and to the cognate receptor for the displayed polypeptide. We have found that this

holds true for retroviral vectors displaying epidermal growth factor (EGF). However, depending on its precise nature, its propensity to oligomerise and its mode of linkage to the SU glycoprotein, the displayed polypeptide may sterically hinder the interaction between the N-terminal domain of SU and the natural virus receptor.

Modification of retrovirus tropism by N-terminally extended SU glycoproteins

When different receptor-binding domains were displayed on MLV retroviral vectors as N-terminal extensions of their intact SU glycoproteins, it was found that host range could be extended or restricted by the displayed ligand (Cosset *et al.* 1995 J. Virol. *69*, 6314-6322). Thus, as a demonstration of host range extension, murine ecotropic vectors displaying the RAM-1 receptor-binding domain from 4070A SU were able to bind and infect RAM-1-positive human cells. In contrast, as a demonstration of host range restriction, ecotropic and amphotropic vectors displaying EGF could bind to EGF receptors but were thereafter sequestered into a non-infectious entry pathway, giving greatly reduced titres on EGF receptor-positive cells, but normal titres on EGF receptor-negative cells. EGF receptor-negative cells, which were fully susceptible to the engineered retroviral vector, showed reduced susceptibility when they were genetically modified to express EGF receptors. The reduction in susceptibility was in proportion to the level of EGF receptor expression. Moreover, when soluble EGF was added to competitively inhibit virus capture by the EGF receptors, gene transfer was restored. In this latter example, the engineered vector is capable of binding to the natural virus receptor or to the receptor for EGF; attachment to the natural virus receptor leads to infection of the target cell, whereas the attachment to the EGF receptor does not lead to infection of the target cell. Where the target cell expresses both species of receptor, the two binding reactions (4070A envelope protein to RAM-1, and EGF to EGF receptor) proceed in competition and the infectivity of the virus for the target cells is reduced in proportion to the efficiency with which the EGF-EGF receptor binding reaction competes virus away from RAM-1.

The degree to which gene transfer can be inhibited by this mechanism depends on the relative affinities of the two binding reactions (envelope protein to natural receptor and non-viral ligand to its cognate receptor), the relative densities of the two receptors on the

target cell surface, and the relative densities of the nonviral ligand and the intact envelope protein on the viral surface. Inhibition of gene transfer is additionally influenced by intrinsic properties of the receptor for the non-viral ligand, such as the distance it projects from the target cell membrane, its mobility within the target cell membrane and its half life on the cell surface after engagement of ligand.

Steric hindrance of the interaction between the N-terminal domain of SU and the natural virus receptor provides an alternative mechanism whereby polypeptides displayed as N-terminal extensions of SU can restrict retroviral host range. For example, chimaeric envelopes displaying the N-terminal domain from 4070A MLV SU as an N-terminal extension of Moloney MLV SU can apparently bind to RAM-1 (the receptor for 4070A SU) but not to ecoR (the receptor for Moloney SU); it may be possible that the displayed domains from 4070A SU may form a trimeric cap over the Moloney SU trimer, completely masking its receptor binding sites. If this model is correct, then it should also be possible to generate chimaeric envelopes in which the receptor binding sites of the intact 4070A SU glycoprotein (through which the virus attaches to human cells) are masked by a displayed polypeptide, such as the N-terminal domain of Moloney MLV SU, that does not bind to human cells.

Phage display of cleavable domains

There are certain similarities between retroviral vectors displaying polypeptide ligands as N-terminal extensions of their envelope glycoproteins and filamentous bacteriophage displaying polypeptide ligands as N-terminal extensions of the gene III protein. Libraries of filamentous "substrate phage" displaying cleavable binding domains have recently been used to identify optimal substrates for known proteases (Matthews & Wells 1993 Science 260, 1113; Matthews *et al.* 1994 Protein Science 3, 1197; Smith *et al.* 1995 J. Biol. Chem. 270, 6440). However, filamentous phages do not naturally infect mammalian cells and there has been no demonstration that cleavable domains fused to the gene III protein can influence the tropism of the phages on which they are displayed.

Summary of the Invention

In a first aspect the invention provides a recombinant viral particle capable of infecting a

eukaryotic cell, the viral particle comprising: a substantially intact viral glycoprotein fused, via an intervening linker region, to a heterologous polypeptide displayed on the surface of the particle, which heterologous polypeptide modulates the ability of the viral particle to infect one or more eukaryotic cell types and is cleavable from the viral glycoprotein by a protease acting selectively on a specific protease cleavage site present in the linker region, such that cleavage of the heterologous polypeptide from the viral glycoprotein allows the glycoprotein to interact normally with its cognate receptor on the surface of a target cell.

Such a particle is of considerable benefit in the targeted delivery of nucleic acid sequences, which may be present within the particle, to specific desired target cells, such as is required for gene therapy.

In another aspect the invention provides a nucleic acid construct, comprising a sequence encoding a fusion protein, the fusion comprising a substantially intact viral glycoprotein fused, via an intervening linker region, to a heterologous polypeptide, wherein the fusion protein is capable of being incorporated into a viral particle capable of infecting an eukaryotic cell, and further wherein the heterologous polypeptide modulates the ability of the viral particle to infect one or more eukaryotic cell types, but cleavage of the heterologous polypeptide from the fusion protein allows the viral glycoprotein to interact normally with its cognate receptor on the surface of the eukaryotic cell.

In a further aspect the invention provides a nucleic acid sequence library comprising a plurality of the nucleic acid constructs defined above, wherein at least part of the sequence encoding the intervening linker region is randomised in each construct, such that each construct comprises one of a plurality of different linker regions which are represented in the library.

The invention also provides a library of the viral particles defined above, each particle comprising a single nucleic acid construct from the nucleic acid library defined above.

The term "substantially intact" as used herein is intended to refer to a viral glycoprotein

which retains all of its domains so as to conserve post-translational processing, oligomerisation (if any), viral incorporation and fusogenic properties. However, certain alterations (e.g. point mutations, deletions, additions) can be made to the glycoprotein without significantly affecting these functions, and glycoproteins containing such minor modifications are considered substantially intact for present purposes. In particular, the gluycoprotein may lack a few (e.g. about 1 to 10) amino acid residues, especially at the N terminus, but will otherwise be generally the same size as the wild-type protein and possess substantially the same biological properties as the wild-type protein.

The intervening linker region will preferably be quite short, typically comprising from 4 to 30 amino acid residues, more typically 5 to 10 residues. A short linker is preferred, because this will tend to maximise the modulation of infection effected by the heterologous polypeptide. In certain embodiments, a suitable linker region may be present as a natural part of the heterologous displayed polypeptide.

The viral particle may be any virus capable of infecting one or more eukaryotic cell types, but conveniently will be a viral particle suitable for use in gene therapy, such as an adenovirus or a retrovirus (especially a C-type retrovirus).

The viral glycoprotein will typically comprise a viral envelope glycoprotein, or may be a chimeric polypeptide comprising sequences corresponding to different viral glycoproteins but which, in total, consitute a substantially intact, functional protein.

The heterologous polypeptide may be a short amino acid sequence (say, a peptide of about 10-20 residues, especially if the sequence undergoes oligomerisation, e.g. a leucine zipper peptide sequence) but more typically will comprise 30 or more amino acid residues. Generally, but not essentially, the polypeptide will comprise a functional binding domain. The heterologous polypeptide, when fused to the viral glycoprotein via the linker region, modulates the ability of the viral particle to infect one or more eukaryotic cell types. Specifically, the presence of the heterologous polypeptide serves to inhibit the process of infection of a eukaryotic target cell mediated by the viral glycoprotein. The term "heterologous" is intended to refer to any polypeptide which is not naturally fused or

otherwise bound to the viral glycoprotein.

The heterologous polypeptide may or may not possess specific binding affinity for a surface component of a target cell. In one embodiment, the heterologous polypeptide has affinity for a cell surface component, binding to which will not lead to infection of the cell by the virus. Within this general embodiment, a variety of different examples (each with different properties) can be envisaged. In one example, a eukaryotic cell expresses a receptor for the viral glycoprotein (binding to which allows the virus to infect the cell) and a non-permissive receptor for the heterologous polypeptide, with inhibition of infection resulting simply from competition between the viral glycoprotein and the heterologous polypeptide for binding to their respective receptors on the target cell. In a different example, the conformational arrangement of the respective receptors and their ligands is such that binding of the heterologous polypeptide to its receptor causes steric hindrance, such that binding of the viral glycoprotein to its receptor, or fusion of the virus and the cell, is blocked.

In a second embodiment, the heterologous polypeptide does not bind to a non-permissive receptor on the target cell, but the presence of the heterologous polypeptide serves to create steric hindrance sufficient to prevent binding of the viral glycoprotein to its receptor, or may allow binding to occur but inhibits subsequent fusion of the viral particle with the target cell, such that infection of the cell by the viral particle is inhibited at the binding and/or fusion stage.

In a particular embodiment the heterologous polypeptide is capable of forming oligomers when displayed on the surface of the viral particle. Typically the oligomer will be a dimer or, more preferably, a trimer. Such oligomerisation may allow for efficient inhibition of the interaction between the substantially intact viral glycoprotein and its receptor, which inhibition may be removed by proteolytic cleavage of the oligomerised heterologous polypeptide from the viral glycoprotein. The intervening linker may also undergo oligomerisation.

Where the viral glycoprotein is itself capable of forming oligomers (e.g. retroviral env

protein), it is preferred that the heterologous polypeptide oligomerises with the same stoichiometry as that of the viral glycoprotein. Vascular endothelial growth factor (VEGF) and tumour necrosis factor (TNF) are both proteins which are known to oligomerise and have high affinity for cell surface ligands. Effective (oligomer-forming, and preferably ligand-binding) portions of these proteins may be particularly suitable for use as heterologous polypeptides in accordance with the present invention.

In the present invention the heterologous polypeptide is cleavable from the viral glycoprotein by the selective action of a protease (i.e. a molecule capable of cleaving a peptide bond) which cleaves the linker region at a protease cleavage site. The cleavage site represents a unique peptide sequence not present, or at least not accessible to the protease, in the viral glycoprotein, although a similar site may be present in the heterologous polypeptide (this is generally preferably avoided, as proteolytic attack on the heterologous polypeptide may affect its functioning). The size, and number, of the protease cleavage sites in the linker region may be varied with advantage. Thus, for example, the presence of two or more cleavage sites, recognised by the same or by respective proteases could facilitate cleavage, whilst the use of one long cleavage site will tend to enhance specificity of cleavage.

Large numbers of specific proteases, and the cleavage sites they recognise, are known to those skilled in the art (see, for example, Vassalli & Pepper 1994 Nature *370*, 14-15, and references cited therein). Proteases are involved in a number of physiological and/or pathological processes, such as tissue remodelling, wound healing, inflammation and tumour invasion, and such proteases would be of use in the present invention. Specific classes of protease which would be of use include: serine proteases (such as plasminogen/plasmin enzymes); cysteine proteases; and matrix metalloproteinases (MMPs) of various types, (such as Gelatinase A and membrane-type MMP [or MT-MMP]).

The protease which serves to cleave the heterologous polypeptide from the viral glycoprotein is preferably selectively secreted by the cell to which it is desired to target the viral particle, or at least the tissue in which the target cell is located. It is preferred that the protease will be secreted only by cells of the target cell type or, less preferably,

only by cells (other than the target cells) remote from the tissue containing the target cell. This confers an extra degree of specificity, which is desirable when the particle is used for targeted gene delivery. Thus the present invention allows for two-step targeting, in which a first level of specificity may be imposed by the heterologous polypeptide (e.g. with specific affinity for a ligand on the surface of the target cell), and a second level of specificity may be imposed by selective cleavage of the heterologous polypeptide by proteases secreted by, or in the same tissue as, the target cell. Alternatively, the relevant protease may be added exogenously, such that if the viral particle is used for targeted gene delivery in a patient, the protease may be administered (e.g. by injection) to the tissue in which the target cell is located.

Accessibility of the protease cleavage site to the relevant protease (i.e. that which recognises and cleaves the site) may also be varied. It has been found by the present inventors that use of a short intervening linker region (e.g. 5 amino acid residues) tends to restrict accessibility of the cleavage site, and use of a larger linker region (e.g. 15 to 20 residues) tends to increase accessibility of the cleavage site. This phenomenon is presumably due to seric hindrance of the cleavage site due to the proximity of the viral glycoprotein and/or the heterologous polypeptide. Accordingly, it should also be possible to modify accessibility of the cleavage site, as desired, by varying the size of the heterologous polypeptide.

In one embodiment, the cleavage site is accessible to the relevant protease before the viral particle becomes bound to an eukaryotic cell, whilst in an alternative embodiment the cleavage site is inaccessible to the protease until the viral particle has become bound to a eukaryotic cell. In this latter embodiment, the cleavage site may be made accessible by a conformational change occurring as a result of binding of the heterologous polypeptide to its cognate receptor. Alternatively, the viral glycoprotein binding to its cognate receptor may make the cleavage site accessible, cleavage of the heterologous polypeptide then allowing fusion of the viral particle to the eukaryotic target cell.

As indicated above, in another aspect the invention provides for a method of selectively delivering a nucleic acid to a target eukaryotic cell present among non-target cells,

comprising: administering to the target and non-target cells a recombinant viral particle capable of infecting eukaryotic cells, the particle comprising the nucleic acid to be delivered, and a fusion protein comprising a substantially intact viral glycoprotein fused, via an intervening linker region, to a heterologous polypeptide displayed on the surface of the particle, which heterologous polypeptide modulates the ability of the particle to infect one or more eukaryotic cell types and being cleavable from the glycoprotein by a protease acting selectively on a specific protease cleavage site present in the linker region, such that cleavage of the heterologous polypeptide from the glycoprotein occurs preferentially at, or in the vicinity of, the target cell and allows the viral glycoprotein to interact normally with its cognate receptor on the surface of the target cell.

The method may be performed *in vitro*, for example to deliver a lethal nucleic acid to fibroblasts in tissue culture, which cells often outgrow a slower-growing, more differentiated cell type in culture. Alternatively, the method may be performed as a method of gene therapy, *in vivo* or may be performed *ex vivo*, on cells which are then re-introduced into a human or animal subject. Preferential cleavage of the protease cleavage site may occur only when the viral particle is bound to the target cell, or when the viral particle is adjacent to the target cell and thus exposed to a protease secreted by the target cell. It may well be preferred to add the relevant protease exogenously, after administration of the viral particle, so as to ensure sufficient concentration of the protease and as another aid to specificity of delivery (by local administration of the protease). It is already known that some proteases may be safely given *in vivo* (e.g. those enzymes, such as urokinase, streptokinase and tPA, given to patients with myocardial infarcts).

The invention also provides, in a further aspect, a method of screening nucleic acid sequences for those which encode an amino acid sequence which may or may not be cleaved by a protease. As already mentioned, many viral envelope glycoproteins are processed through the cellular export pathway of the eukaryotic cell in which they are synthesised, generally leading to cleavage, which cleavge is essential for production of an infectious viral particle.

The invention therefore provides a method of screening nucleic acid sequences for those

which encode an amino acid sequence which may or may not be cleaved by a protease present in the export pathway of an eukaryotic cell, comprising: causing the expression of a plurality of nucleic acid sequences in eukaryotic cells, each sequence encoding a substantially intact viral glycoprotein fused to a heterologous polypeptide via a randomised intervening linker region, the presence of the heterologous polypeptide serving to inhibit the (binding or fusion) interaction of the viral glycoprotein with its cognate receptor, and wherein each nucleic acid sequence further comprises a packaging signal allowing for viral incorporation, such that those intervening linkers which are recognised by a protease present in the export pathway of the eukaryotic cells will allow for cleavage of the heterologous polypeptide from the viral glycoprotein, resulting in the production of an infectious viral particle; and recovering those nucleic acid sequences directing the expression of such cleavable linker regions from an infected cell.

Nucleic acid sequence determination may optionally be performed, to deduce those amino acid sequences which are recognised by an export protease.

A modification of the above method will allow for the screening of nucleic acid sequences for those which encode an amino acid sequence which may or may not be cleaved by a protease present in the eukaryotic cell import pathway. As explained above, the presence of a heterologous polypeptide may, in some embodiments, still allow for binding of the viral glycoprotein to its cognate receptor, but will prevent fusion of the viral particle with the eukaryotic cell to which it is bound. Cleavage of the heterologous polypeptide by a protease in the cellular import pathway will then allow infection of the cell.

Thus, in a further aspect the invention provides for a method of screening nucleic acid sequences for those which encode an amino acid sequence which may or may not be cleaved by a protease, comprising: causing the expression of a plurality of nucleic acid sequences in eukaryotic cells, each sequence encoding a substantially intact viral glycoprotein fused to a heterologous polypeptide via a randomised intervening linker region, the presence of the heterologous polypeptide serving to inhibit the fusion of a viral particle with a eukaryotic cell to which it is bound, and wherein each nucleic acid sequence further comprises a packaging signal allowing for viral incorporation; enriching

the viral particles so produced for those which retain the heterologous polypeptide (and so are non-infectious); and contacting the enriched particles with a susceptible eukaryotic cell comprising, or in the presence of, a protease such that those intervening linkers which are recognised by the protease will allow for cleavage of the heterologous polypeptide from the viral glycoprotein, resulting in productive infection of the eukaryotic cell; and recovering those nucleic acid sequences directing the expression of such cleavable linker regions from the infected cell. As above nucleic acid sequence determination may optionally be performed to allow deduction of the corresponding amino acid sequences.

The enrichment step is required because of the possibility that the heterologous polypeptide may be cleaved from the viral glycoprotein by an export pathway protease during synthesis of the particles. A number of possible enrichment techniques will be readily apparennt to those skiled in the art with the benefit of the present teaching. For example, prior to infection of the susceptible cells, the viral particles could be subjected to an affinity enrichment technique - the particles could be passed through an antibody affinity column, wherein the antibody has affinity for the heterologous polypeptide. Those particles which retain the heterologous polypeptide will be bound to the column, whilst those in which the heterologous polypeptide was cleaved during export from the producing cell will pass straight through the column. After washing, the bound particles may be eluted (e.g. by competition with free heterologous polypeptide, or the part thereof recognised by the antibody, or by alteration of pH or other factors) and then used to infect the susceptible "indicator" cells.

The invention will now be further described by way of illustrative examples, and with reference to the accompanying figures, in which:

Figure 1 is a schematic representation of retroviral vector constructs coding for chimeric envelopes;

Figures 2 and 3 are photographs of Western blots demonstrating viral incorporation of certain chimeric polypeptides and their sensitivity to Factor Xa protease;

Figure 4 is a photograph showing the infectivity of various β-galactosidase transducing viruses on target cells with or without Factor xa treatment, as judged by assay on X-gal containing plates;

Figure 5 is a schematic representation of how two-step targeting of gene delivery might be achieved using the present invention;

Figure 6A is a photograph of a Western blot demonstrating viral incorporation of certain chimeric polypeptides and their sensitivity to Factor Xa protease;

Figure 6B is a bar chart illustrating the infectivity of certain recombinant viruses in the presence or absence of Factor Xa;

Figure 7 is a schematic representation of retroviral vector constructs coding for chimeric envelopes;

Figure 8A is a photograph of two Western blots, the upper one comparing electrophoretic mobility of various chimeric polypeptides, the lower one comparing the amount of protein present;

Figure 8B is a photograph of a Western blot comparing the sensitivity to Factor Xa protease of various chimeric polypeptides;

Figure 8C is a photograph of a Western blot comparing processing of certain chimeric polypeptides;

Figure 9 is a panel of photographs comparing the growth of of a recombinant virus on NIH 3T3 and A431 cells, with or without Factor Xa treatment;

Figure 10 is a schematic representation of retroviral vector constructs coding for chimeric envelopes;

Figure 11 shows three Tables, A, B and C, illustrating the titre (in enzyme forming units, "e.f.u.") of various recombinant viruses on NIH 3T3 or A431 cells in the absence (-) or presence (+) of Factor Xa protease;

Figure 12 is a schematic representation of retroviral vector constructs coding for chimeric envelopes;

Figure 13A is a photograph of a Western blot demonstrating viral incorporation of various chimeric polypeptides;

Figure 13B is a photograph of a Western blot comparing the sensitivity of various chimeric polypeptides in the presence (+) or absence (-) of pro-gelatinase A, with (+) or without (-) pre-activation of the protease by p-aminophenylmercuric acetate (APMA);

Figure 14 is a bar chart showing how infectivity of a recombinant virus is dependent upon concentration of pro-gelatinase A;

Figure 15 is a bar chart comparing the infectivity of three different recombinant viruses on HT 1080 or A431 cells;

Figure 15A is a photograph comparing the growth of a recombinant virus on HT 1080 or A431 cells;

Figure 16 is a panel of four photographs (I, II, III and IV) comparing the infectivity of various viruses on HT 1080 (H) or A431 (A) cells;

Figure 17 is a photograph of a gel for detection of gelatinolytic activity; and

Figures 18 and 19 are schematic representations of retroviral vector constructs coding for chimeric envelopes.

EXAMPLES

Example 1

Summary

Tropism-modifying binding domains were anchored to murine leukaemia virus (MLV) envelopes via factor Xa-cleavable linkers to generate retroviral vectors whose tropism could be regulated by factor Xa protease. The binding domains could not be cleaved from vector particles by factor Xa when the linker was fused to amino acid +7 of Moloney MLV SU but could be efficiently cleaved when fused to amino acid +1 of Moloney or 4070A MLV SU glycoproteins. Vectors displaying a cleavable EGF domain were selectively sequestered on EGF receptor-expressing cells, but their infectivity was fully restored when the EGF domain was cleaved from the vector particles with factor Xa. Partial restoration of infectivity was observed when only a fraction of the envelope proteins were cleaved. Conversely, vectors that displayed a cleavable RAM-1 binding domain fused to Moloney MLV SU had an expanded host range that was reversible upon treatment with factor Xa. It is suggested that retroviral vectors with engineered binding specificities whose tropism is regulated by exposure to specific proteases may facilitate novel strategies for targeting retroviral gene delivery.

Introduction, results, and discussion

MLV-derived retroviral vectors are versatile gene delivery vehicles whose host range can be varied by incorporation of different envelope spike glycoproteins (Miller, 1992 Curr. Top. Microbiol. Immunol. *158*, 1; Vile & Russell, 1995 British Medical Bulletin. *51*, 12; Weiss, in Retroviridae, J. Levy, Ed. (Plenum Press, 1993), pp. 1-108). Retroviral envelope spike glycoproteins mediate virus attachment to specific receptors on the target cell surface and subsequently trigger fusion between the lipid membranes of virus and host cell. The envelope spike glycoproteins of murine leukaemia viruses (MLVs) are homotrimers in which each of the three heterodimeric subunits comprises a large extraviral glycoprotein moiety (SU) attached at its C-terminus to a smaller transmembrane polypeptide (TM) that anchors the complex in the viral membrane (August *et al.*, 1974 Virology *60*, 595; Ikeda *et al.*, 1975 J. Virol. *16*, 53; Kamps *et al.*, 1991 Virology *184*, 687). SU consists of two domains connected by a proline-rich hinge, the N-terminal domain conferring receptor specificity and exhibiting a high degree of conservation between MLVs with different host ranges (Battini *et al.*, 1992 J. Virol. *66*, 1468; Morgan

et al., 1993 J. Virol. *67*, 4712; Battini *et al.*, 1995 J. Virol. *69*, 713). Moloney MLV envelopes confer an ecotropic host range because they attach selectively to a peptide loop in the murine cationic amino acid transporter (CAT-1), found only on cells of mouse and rat origin (Albritton *et al.*, 1989 Cell *57*, 659; Albritton *et al.*, 1993 J. Virol. *67*, 2091). 4070A MLV envelopes attach to an epitope on the ubiquitous RAM-1 phosphate symporter that is conserved throughout many mammalian species and confer an amphotropic host range (Miller *et al.*, 1994 Proc. Natl. Acad. Sci. U.S.A. *91*, 78; VanZeijl *et al.*, 1994 Proc. Natl. Acad. Sci. U.S.A. *91*, 1168; Kavanaugh *et al.*, 1994 Proc. Natl. Acad. Sci. U.S.A. *91*, 7071). Thus, retroviral vectors with 4070A envelopes infect human cells promiscuously whereas vectors with Moloney envelopes fail completely to infect human cells.

Cell-selective retroviral gene delivery has recently been achieved by engineering new binding domains into the envelope glycoproteins of retroviral vectors (Valsesia-Wittmann *et al.*, 1994 J. Virol. *68*, 4609; Kasahara *et al.*, 1994 Science *266*, 1373; Chu & Dornburg, 1995 J Virol. *69*, 2659; Nikunj *et al.*, 1995 Proc. Natl. Acad. Sci. USA *92*, 7570; Cosset *et al.*, 1995 J. Virol. *69*, 6314). When different receptor-binding domains were displayed on MLV retroviral vectors as N-terminal extensions of their intact SU glycoproteins (Russell *et al.*, 1993 Nucl. Acids Res. *21*, 1081), it was found that host range could be extended or restricted by the displayed ligand (Cosset *et al.*, 1995 J. Virol. *69*, 6314). Thus, ecotropic vectors displaying a RAM-1 receptor-binding domain from 4070A SU were able to infect RAM-1-positive human cells whereas amphotropic vectors displaying epidermal growth factor (EGF) could bind to EGF receptors but were thereafter sequestered into a noninfectious entry pathway, giving greatly reduced titres on EGF receptor-positive cells, but normal titres on EGF receptor-negative cells. In the current study, we have explored the possibility of generating retroviral vectors whose engineered tropism can be regulated by specific proteases.

Initially, we inserted a short factor Xa protease-sensitive linker (amino acid sequence IEGR), (Lottenberg *et al.*, 1981 Methods Enzymol. *80*, 341), into a previously described EGF-MLV envelope chimaera (EMo7) in which the EGF domain was fused to amino acid +7 in Moloney MLV SU by a short linker containing three alanines. The Xa cleavage

signal was inserted between the alanine linker and amino acid +7 of Moloney SU to give the construct EXMo7, described below and illustrated in Figure 1. In Figure 1, the general format for all of the constructs is shown diagrammatically and the sequence surrounding the site of fusion between the displayed ligand (EGF or N-terminal binding domain of 4070A-MLV) and the MLV envelope protein (Moloney or 4070A) is shown in detail for each of the constructs. Beside each construct is a schematic representation of the N-terminal region of the expressed envelope glycoprotein monomer; Open circles indicate N-terminal receptor-binding domain of the (ecotropic) Moloney MLV SU glycoprotein, filled squares indicate the N-terminal receptor binding domain of the (amphotropic) 4070A MLV SU glycoprotein, grey triangles represent EGF, and factor Xa cleavage sites are denoted with arrows. LTR: long terminal repeat, L: envelope signal peptide, p: polyadenylation sequence. The *Not*I cloning site is also shown.

The chimaeric envelopes and a control ecotropic (Moloney) envelope were expressed in TELCeB6 cells which express MLV gag-pol core particles and an nlsLacZ retroviral vector (Cosset *et al.*, 1995 J. Virol. *69*, 7430-7436). Virus-containing supernatants from the transfected TELCeB6 cells were harvested, filtered (0.45μm), digested with 0 or 4 μg/ml factor Xa protease for 90 minutes and ultracentrifuged to pellet the viral particles. Retroviral particles incorporating chimaeric envelopes were analyzed by Western immunoblotting (Figure 2) before (-) or after (+) treatment with factor Xa protease. Lanes A, B and C were loaded with pelleted retroviral vectors incorporating Mo, EXMo1, and EXMo7 envelopes, respectively. The different envelope expression constructs were transfected (as described in Sambrook *et al.*, Molecular cloning, A laboratory manual, (Cold Spring Habour, N.Y., 1989) pp. 16.33-16.36) into TELCeB6 packaging cells and stable phleomycin (50 μg/ml) resistant colonies were expanded and pooled. Cells were grown in DMEM supplemented with 10% fetal calf serum and when confluent transferred from 37°C to 32°C and incubated for 72 hrs. Supernatants containing retroviral particles were harvested after overnight (16 hrs) incubation in 10 mls serum-free DMEM at 32°C and filtered (0.45μm) before being incubated with 0 or 4 μg/ml of factor Xa (Promega) for 90 minutes at 37°C in the presence of 2.5 mM $CaCl_2$. The supernatants were centrifuged at 30 000 rpm in a SW40 rotor (Beckman) for 1 hour at 4°C and the pelleted viral particles were resuspended in 100μl phosphate buffered saline. 20μl of each sample

was separated on a 10% polyacrylamide gel under reducing conditions (Laemmli, Nature (London, *277*, 680 (1970)) followed by transfer of the proteins onto nitrocellulose paper. The SU proteins were detected as previously described (Cosset *et al.*, 1995 J. Virol. *69*, 6314) using specific goat antibodies raised against Rausher murine leukaemia virus envelope glycoproteins (Quality Biotech Inc, USA) followed by Horseradish peroxidase-conjugated rabbit anti-goat antibodies (DAKO Denmark) and developed using an enhanced chemiluminescence kit (Amersham Life Science).

EGF was not cleaved from the EXMo7 envelope by factor Xa (Fig. 2, lane C), suggesting that the cleavage site was not accessible to the protease when inserted in this position.

We therefore made new constructs, EMo1 and EXMo1 (described below), coding for chimaeric envelopes in which EGF is fused to amino acid +1 (rather than +7) of Moloney SU by a linker comprising 3 alanines, or 3 alanines and the IEGR factor Xa cleavage site (see Figure 1). EMo1 and EXMo1 chimaeric envelopes were incorporated into virions and analysed on immunoblots after treatment with 0 or 4 μg/ml factor Xa protease for 90 minutes. Figure 2 shows that EXMo1 envelopes were cleaved by factor Xa to yield an SU cleavage product whose mobility was indistinguishable from unmodified Moloney SU. Control EMo1 envelopes which lack the factor Xa cleavage site were not cleaved. These results indicate that the precise positioning of the IEGR peptide in the chimaeric envelopes is important for its optimal recognition and cleavage by factor Xa.

We have previously demonstrated EGF receptor-mediated host range restriction of retroviral vectors displaying chimaeric envelopes in which EGF was fused to amino acid +5 of 4070A SU by a short (AAA) linker (Cosset *et al.*, J. Virol. *69*, 1995, cited above). Retroviruses displaying these chimaeric envelopes could bind to EGF receptors but were thereafter sequestered into a noninfectious entry pathway, giving greatly reduced titres on EGF receptor-positive cells, but near-normal titres on EGF receptor-negative cells.

We therefore constructed plasmids EA1 and EXA1 (described below) coding for chimaeric envelopes in which EGF is fused to amino acid +1 of 4070A SU by AAA or AAAIEGR linkers respectively (see Figure 1).

DNA Constructs

The expression plasmids FBMoSALF and FB4070ASALF (described by Cosset *et al.*, 1995 J. Virol. *69*, cited above) coding for unmodified Moloney and 4070A MLV envelopes are referred to in the text as Mo and A respectively. Construction of EA, EMo7 (previously called EMO) and AMO expression plasmids was also described by Cosset *et al.*, (cited above).

To generate EXo7, EMo1 and EXMo1, PCR primers NotXMo7Back, NotMo1Back and NotXMo1Back (respectively) were used with primer envseq7 to amplify modified envelope fragments from Mo (FBMoSALF) which were digested with *Not*I and *Bam*HI and cloned into the *Not*I/*Bam*HI-digested backbone of EMo7.

To generate EA1 and EXA1, PCR primers NotA1Back and NotXA1Back (respectively) were used with primer 4070Afor to amplify modified envelope fragments from A (FB4070ASALF) which were digested with *Not*I and *Bam*HI and cloned into the *Not*I/*Bam*HI-digested backbone of EA.

Finally, the AMo1 and AXMo1 constructs (referred to below) were generated by cloning the *Nde*I-*Not*I fragment from AMO into the *Nde*I/*Not*I-digested backbones of EMo1 and EXMo1, respectively. The correctness of all constructs were confirmed by DNA sequencing.

Oliogonucleotides used (with restriction sites underline) were:

NotXMo7Back, 5'-GCA AAT CTG CGG CCG CAA TCG AGG GAA GGC CTC ATC AAG TCT ATA ATA TCA CC (Seq ID No. 1);

NotMo1Back, 5'-GCA AAT CTG CGG CCG CAG CTT CGC CCG GCT CCA GTC C (Seq ID No. 2);

NotXMo1Back, 5'-GCA AAT CTG CGG CCG CAA TCG AGG GAA GGG CTT CGC CCG GCT CCA GTC C-3' (Seq ID No. 3);

NotA1Back 5'GCA AAT CTG CGG CCG CAA TGG CAG AGA GCC CCC ATC-3' (Seq ID No. 4);

NotXA1Back 5'-GCA AAT CTG CGG CCG CAA TCG AGG GAA GGA TGG CAG AGA GCC CCC ATC-3' (Seq ID No. 5);

envseq7, 5'-GCC AGA ACG GGG TTT GGC C-3' (Seq ID No. 6);

4070Afor, 5'-CTG CAA GCC CAC ATT GTT CC-3' (Seq ID No. 7).

Figure 3 shows that the IEGR sequence in the interdomain linker of the expressed EXA1 envelopes was correctly recognized and cleaved by factor Xa whereas there was no cleavage of control EA1 envelopes. Referring to Figure 3 (an immunoblot of the recombinant amphotropic retroviral particles before (-) or after (+) treatment with factor Xa protease): lanes A, B and C were loaded with pelleted retroviral vectors incorporating A, EA1 and EXA1 envelopes, respectively. The analysis was performed as described above for Figure 2.

We then titrated vectors incorporating EA1 and EXA1 chimaeric envelopes on EGF receptor-negative and EGF receptor-positive human cell lines as follows: EGF receptor-expressing cell lines A431 (ATCC CRL1555), HT1080 (ATCC CCL121), and EJ (Bubenik, *et al.*, 1973 Int. J. Cancer *11*, 765) were grown in DMEM supplemented with 10% fetal calf serum (Gibco-BRL) at 37°C in an atmosphere of 5% CO_2. Jurkat T cells (ATCC CRL8805) were grown in RPMI supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2. For infections, target cells were seeded at 2 x 10^5 cells/well in six-well plates and incubated at 37°C overnight. Producer cell supernatants containing β-galactosidase-transducing retroviruses were filtered (0.45 μm) after overnight incubation at 32°C in serum free medium. Supernatant dilutions in 2.5 ml serum-free medium were incubated with target cells for 2 hours in the presence of 8 μg/ml polybrene. The retroviral supernatant was then removed and the cells were incubated with regular medium for 48-72 hours. X-Gal staining for detection of β-galactosidase activity was performed as previously described (Takeuchi *et al.*, 1994 J. Virol. *68*, 8001). Viral titre

(enzyme forming units/ml) was calculated by counting blue stained colonies microscopically with the use of a grid place underneath the 6 well plates.

Both vectors incorporating EA1 or EXA1 envelopes could infect EGF receptor-negative Jurkat cells but were selectively sequestered on EGF receptor-expressing human cells, although EXA1 was sequestered less completely than EA1 (Table 1). When soluble EGF was added as competitor to prevent the vectors from binding to EGF receptors their infectivity on EGF receptor positive cells could be fully restored (Table 1), confirming that sequestration was mediated specifically through binding of the engineered envelopes to EGF receptors.

We then tested whether the restricted host range conferred by EXA 1 envelopes could be extended (i.e. revert to amphotropic) upon cleavage of the displayed EGF domain. Vectors incorporating EA1 or EXA1 envelopes were treated with increasing doses of factor Xa and titrated on EGF receptor-expressing A431 cells (Table 2). Complete cleavage of the fused EGF domain with 4 μg/ml factor Xa for 90 minutes (Figure 3) completely restored the infectivity of vectors with EXA1 envelopes but had no effect on the infectivity of vectors carrying EA1 envelopes. Partial restoration of vector titre was seen at lower concentrations of factor Xa indicating that the vector particles could recover a low level of infectivity when only a fraction of their envelope proteins were cleaved. These data provide further evidence that retroviral vectors displaying EGF are competitively sequestered by EGF receptors, and show that their tropism can be regulated by a specific protease that cleaves the EGF domain from the viral surface.

Factor Xa protease is capable of binding directly to procoagulant phospholipid on the surface of an enveloped virus (Pryzdial & Wright, 1994 Blood *84*, 3749-3757) and might therefore go on to become stably associated with phospholipid in the engineered vector particles after cleaving their EXA1 envelopes. A control experiment was therefore performed to confirm that the restoration of infectivity of vectors incorporating EXA1 envelopes on A431 cells was due to cleavage of EGF, and not mediated by particle-associated factor Xa protease. We therefore constructed plasmid AXMo1 and a control plasmid AMo1 (described above), coding for chimaeric envelopes in which the

RAM-1 receptor binding domain is fused to amino acid +1 of Moloney SU by a factor Xa protease-cleavable (AAAIEGR) or non-cleavable (AAA) linker (Figure 1). As expected, vectors incorporating AMo1 and AXMo1 chimaeric envelopes could bind to RAM-1 allowing targeted infection of a variety of human cell lines, and the IEGR sequence in the interdomain linker of the AXMo1 envelope was correctly recognized and cleaved by factor Xa (data not shown). We therefore tested whether the extended host range conferred by AXMo1 envelopes could be restricted (i.e. revert to ecotropic) upon cleavage of the displayed 4070A domain. Treatment with 4 μg/ml factor Xa for 90 minutes selectively destroyed the infectivity of vectors with AXMo1 envelopes on human cells but did not reduce their infectivity on mouse cells (Table 2). Vectors carrying AMo1 envelopes were unaffected by the protease treatment. These data confirm that the extended tropism of retroviral vectors displaying AMo envelopes is due to the displayed 4070A domain and show that the restoration of infectivity of vectors incorporating EXA1 envelopes on A431 cells was due to cleavage of EGF, and not mediated by particle-associated factor Xa protease.

In summary, we have generated retroviral vectors displaying cleavable binding domains that are anchored to the viral envelope glycoprotein by a linker that acts as a substrate for factor Xa protease. The displayed binding domains confer novel host range properties upon the vectors and these host range alterations are reversible upon treating the vectors with factor Xa. In principle it should be possible to use linkers that are substrates for proteases other than factor Xa in conjunction with binding domains that recognise cell

Table 1.

Infection of human cell lines with retroviral vector particles displaying EGF-4070A chimaeric envelopes.

	Titre (CFU/ml) against cell line:				
	A431	HT1080	EJ	EJ	Jurkat
EGF-receptor*	+	+	+	+	-
EGF:†	-	-	-	+	-
Constructs‡					
A	10^0	10^6	10^6	10^6	10^5
EA1	5	10	30	2×10^5	10^4
EXA1	200	100	310	10^5	10^4

* EGF-receptor status determined by FACS analysis.

† + indicates incubation of cells with retroviral vectors in the presence of 1 μM human EGF (R&D systems, UK).

Table 2. Regulation of vector tropism by factor Xa protease.

	Titre (CFU/ml) against cell line:					
	NIH3T3	NIH3T3	A431	A431	A431	A431
FXa (μg/ml):*	0	4	0	0.0156	0.25	4
Constructs‡						
Mo	10^6	10^6	0	0	0	0
A	10^6	10^6	10^6	10^6	10^6	10^6
EA1	6×10^5	3×10^5	10^2	10^2	10^2	10^2
EXA1	10^6	2×10^6	**1×10^3**	**4×10^3**	**6×10^4**	**5×10^5**
AMo1	2×10^5	2×10^5	10^3	10^3	10^3	10^3
AXMo1	5×10^5	10^6	**5×10^3**	**5×10^3**	**2×10^3**	0

* Filtered supernatants containing β-galactosidase-transducing retroviruses were preincubated with various concentrations (0, 0.0156, 0.25 and 4 μg/ml) of factor Xa (Promega) for 90 minutes at 37°C with 2.5 mM added $CaCl_2$. The treated supernatants were then added to the target cells and the viral titres were determined as described in (12).

surface receptors other than those described above. Retroviral vectors with engineered binding specificity whose tropism is regulated by exposure to specific proteases may facilitate novel strategies for targeting retroviral gene delivery.

Example 2

The inventors sought to establish whether vectors incorporating EXA1 envelopes would recover their infectivity on EGF receptor-positive cells upon cleavage of their displayed EGF domain. Vectors incorporating EA1 or EXA1 envelopes were therefore treated with factor Xa and titrated on EGF receptor-expressing A431 cells. Complete cleavage of the fused EGF domain with 4 μg/ml factor Xa for 90 minutes completely restored the infectivity of vectors with EXA1 envelopes but had no effect on the infectivity of vectors carrying EA1 envelopes (Figure 4). Figure 4 illustrates factor Xa-mediated infection of A431 cells with chimaeric EGF-4070A MLV vector particles. Filtered supernatants containing β-galactosidase-transducing retroviruses (A, EA1, or EXA1) were preincubated with 0 (-) or 4 (+) μg/ml concentrations of factor Xa (Promega) for 90 minutes at 37°C with 2.5 mM added $CaCl_2$. The treated supernatants were then used for target cell transduction, as described above. X-gal-stained plates were photographed without magnification.

Partial restoration of vector titre was seen a lower concentrations of factor Xa (Table 2) indicating that the vector particles could recover a low level of infectivity when only a fraction of their envelope proteins were cleaved. These data provide further evidence that retroviral vectors displaying EGF are competitively sequestered by EGF receptors, and show that their tropism can be regulated by a specific protease that cleaves the EGF domain from the viral surface.

In the two step targeting strategy outlined above, cleavage of the chimaeric envelope is preceded by its attachment to the target cell via the engineered binding domain. Therefore, to determine whether EGF receptor-bound vector particles that were cleaved at the cell surface could go on to infect their target cells, we loaded the vectors onto EGF receptor-positive A431 cells and EJ cells, washed the cells, and then treated them with factor Xa protease. Table 3 shows that, when sequestered onto EGF receptors and then

cleaved by factor Xa protease, the vectors incorporating EXA1, but not EA1 envelopes, proceeded to infect their target cells.

The availability of a targetable, injectable vector would greatly facilitate the development of gene therapy approaches requiring direct *in vivo* gene delivery to selected target tissues. In this report we have demonstrated the feasibility of a novel two step-targeting strategy which may allow the generation of retroviral vectors engineered to infect cells expressing specific receptor/protease combinations. There are many membrane-associated proteases

Table 3. Factor Xa protease triggering retroviral infection on the cell-surface of human A431 and EJ cells.

	Titre (CFU/ml) against cell line:			
	A431	A431	EJ	EJ
FXa (μg/ml):*	0	4	0	4
Constructs‡				
A 	3×10^4	3×10^4	1×10^4	1×10^4
EA1 	<5	<5	0	0
EXA1 	4×10^2	1×10^4	<5	3×10^2

* A431 and EJ cells were incubated with 2 ml of filtered supernatant containing β-galactosidase-transducing retroviruses for 1 hr at 4°C. Cells were then washed two times with cold serum-free medium and incubated with 0 or 4 μg/ml of factor Xa (Promega) for 2 hrs at 37°C in serum-free medium. After incubation for 48 hrs with medium supplemented with 10% fetal calf serum the viral titres were determined as described in Table 1.

that may be of interest in this respect such as the proteases that co-operate in degrading the extracellular matrix during tumour invasion (Poustis-Delpont *et al.*, 1992 Cancer Research 52, 3622-3628; Vassalli & Pepper, 1994 Nature *370*, 14-15; Sato *et al.*, 1994 Nature 370, 61-65; and Chen *et al.*, 1995 Breast Cancer Res. Treat. *31*, 217-226); haematopoietic differentiation antigens that are also membrane proteases (Shipp & Look, 1993 Blood *82*, 1058-1070) or the membrane protease that has been implicated in the entry pathway of HIV (Murakami *et al.*, 1991 Biochim. Biophys. Acta *1079*, 79-284).

Libraries of filamentous "substrate phage" displaying cleavable binding domains have recently been used to identify optimal substrates for known proteases (Matthews & Wells, 1993 Science *260*, 1113-1117; Matthews *et al.*, 1994 Protein Science *3*, 1197-1205; and Smith *et al.*, 1995 J. Biol. Chem. *270*, 6440-6449). In principle, it should be possible to generate similar retrovirus display libraries expressing N-terminally extended envelopes with randomised linker sequences. Such libraries might provide the basis for selection strategies designed to identify novel intracellular or membrane-associated proteases or to isolate chimaeric envelopes that target novel cell-specific receptor-protease combinations.

Example 3

Summary

As described above, several polypeptides have now been displayed on retroviral vector particles as N-terminal extensions of their envelope spike glycoproteins. Folding, assembly, transport, viral incorporation, receptor attachment and fusion triggering by the chimaeric envelopes can be variably influenced by the N-terminal polypeptides, depending on their unique structural and functional characteristics. In this example the inventors demonstrate that the RAM-1 binding domain from the homotrimeric 4070A SU glycoprotein can strongly inhibit Rec-1 mediated infection by the homotrimeric Moloney SU glycoprotein when grafted to its N-terminus. It is also shown that short trimeric leucine zipper peptides, but not a monomeric helical peptide, can inhibit RAM-1 mediated infection by the 4070A envelope when fused to its N-terminus. Cleavage signals were engineered into the chimaeric envelopes such that the displayed polypeptides could be

cleaved from the vector particles by addition of factor Xa protease. In all of the envelopes displaying trimeric polypeptides, the steric block to Rec-1 or Ram-1 mediated infection was reversed when the trimeric N-terminal extensions were cleaved from the virally incorporated envelopes. These data suggest that the masking of envelope functions by the inhibitory N-terminal extensions is a consequence of their assembly into a trimeric complex at the tip of the SU glycoprotein trimer to which they were grafted. The implications for retroviral vector targeting are discussed.

MLV-derived retroviral vectors are versatile gene delivery vehicles whose host range properties are determined by membrane glycoproteins which mediate their attachment to specific receptors and subsequently trigger fusion. The envelope glycoproteins of the murine leukaemia virus (MLV) are displayed as a homotrimeric complex on the surface of the virus (Fass *et al.*, Nature Structural Biology *3*:465-469; Kamps *et al.*, Virology *184*:687-694). Each subunit of the trimer consists of two parts, SU and TM. The SU (surface) component is entirely extraviral and is attached to the retrovirus via the smaller TM component, which anchors the complex in the viral membrane (Pinter *et al.*, Virology *91*:345-351). The N-terminal domain of the SU glycoprotein confers receptor specificity and exhibits a high degree of conservation between MLVs with different host ranges (Battini *et al.*, J. Virol. *69*:713-719). Moloney MLV envelopes confer an ecotropic host range because they bind to a murine cationic amino acid transporter (Albritton *et al.*, J. Virol. *67*:2091-2096; Albritton *et al.*, Cell *57*:659-666). 4070A MLV envelopes attach to the RAM-1 phosphate transporter which is conserved throughout many mammalian species, to confer an amphotropic host range (Kavanaugh *et al.*, Proc. Natl. Acad. Sci. USA *91*:7071-7075). After binding to target cell receptors has occurred, the trimeric SU-TM complex is thought to undergo a large conformational rearrangement which triggers the process of fusion between the viral and target cell membranes.

The inventors and their colleagues have been exploring different strategies for targeting the entry of retroviral vectors into selected target cells by engineering new determinants into their SU glycoproteins (Cosset *et al.*, J. Virol. *69*:6314-6322; Nilson *et al.*, Gene Ther. *3*:280-286; Russell *et al.*, Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.; Valsesia-Wittmann *et al.*, J. Virol. *68*:4609-4619; Valsesia-Wittmann *et al.*, J

Virol. *70*:2059-2064). In the preceding examples is described a novel two-step strategy that allows the targeting of retroviral vectors through protease-substrate interactions, in which the retroviral vector attaches to the target cell via an engineered binding domain (step one), whereupon the engineered linker that tethers the virus to the binding domain is cleaved by a specific protease (step two), allowing the virus to go on and infect the target cell. A disadvantage of this two-step targeting strategy, as set forth above, is that whilst dominating the specificity of vector attachment, the cleavable binding domain does not completely block the ability of the SU trimer to attach to its natural receptor on non target cells. The uncleaved vector therefore retains the ability to infect non target cells through the Ram-1 receptor. To overcome this disadvantage, we were interested to develop envelope modifications that would completely inhibit the infectivity of uncleaved vectors but would permit full restoration of infectivity upon exposure to a selected protease.

In the course of experiments (described in this specification) to characterise the ecotropic infectivity of Moloney MLV envelope chimaeras, it was found that a vector displaying the Ram-1 targeted AXMo1 envelope (Nilson *et al.*, Gene Ther. *3*:280-286), could not efficiently infect cells through the ecotropic receptor (Rec-1) unless it was first cleaved by factor Xa protease. To explain this property of the AXMo1 envelope we hypothesised that the displayed RAM-1 binding domain might be forming a trimeric complex at the tip of the Moloney SU glycoprotein trimer to which it was grafted, thereby blocking its Rec-1 binding site. To further test this hypothesis, we grafted oligomerising leucine zipper peptides (Harbury *et al.*, Science *262*:1401-1407) onto 4070A SU glycoproteins and characterised the properties of retroviral vectors incorporating the chimaeric envelopes.

MATERIALS AND METHODS

Plasmid Construction

The unmodified envelopes of 4070A MLV and Moloney MLV were encoded by the expression plasmids FB4070ASALF (A) and FBMoSALF (Mo), respectively (Cosset *et al.*, 1995 J. Virol. *69*, 7430-7436)). The constructs AMo1 and AXMo1, which code for chimaeric envelopes in which the RAM-1 receptor binding domain from 4070A SU is

fused to amino acid +1 of Moloney SU by a factor Xa protease-cleavable (AAAIEGR) or non-cleavable (AAA) linker have been described previously (Nilson *et al.*, Gene Ther. *3*:280-286). Constructs EA1 and EXA1, coding for chimaeric envelopes in which EGF is fused to amino acid +1 of 4070A SU by a linker comprising three alanines, or three alanines and the IEGR factor Xa cleavage site, have also been described (Nilson *et al.*, Gene Ther. *3*:280-286).

To construct vectors displaying helical peptides, plasmids pEGS1XA1 and pEGS3XA1 were first produced in which there is a 12 amino acid (AAAGGGGS**IEGR**, Seq ID No. 8) or 22 amino acid (AAAGGGGSGGGGSGGGGS**IEGR**, Seq ID No. 9) linker, respectively, between the 4070A MLV envelope and the displayed EGF domain. PCR primers NotGS1XA1back and NotGS3XA1back (respectively) were used with primer 4070Afor to amplify modified envelope fragments from EXA1 which were digested with *NotI* and *BamHI* and cloned into the *NotI/BamHI*-digested backbone of EA1.

Figure 7 is a diagramatic representation of plasmid constructs coding for chimaeric envelope glycoproteins in which the helical peptides AA, VL and II were fused to residue +1 of the 4070A MLV SU. The general format is shown diagramatically and the amino acid sequence (single letter code) of the helical peptides and the linkers between these peptides and the SU protein are shown in detail. LTR, long terminal repeat; L, envelope signal peptide. Amino acid residues at the **a** and **d** positions of the heptad repeat are shown in bold.

To generate plasmids pVLXA1, pVLGS1XA1 and pVLGS3XA1, PCR primers Gal4 VLback and Gal4 VLfor were used to produce PCR fragments by priming off each other and then outer primers Gal4back and Gal4for were used to amplify the fragment further. The PCR products were digested with *SfiI* and *NotI* and cloned into the *SfiI/NotI*-digested backbones of EXA1, pEGS1XA1 and pEGS3XA1.

To generate plasmids pAAXA1 and pAAGS3XA1, PCR primers Gal4 AAback and Gal4 AAfor were used to produce PCR fragments by priming off each other and then outer primers Gal4back and Gal4for were used to amplify the fragments further. The PCR

products were digested with *SfiI* and *NotI* and cloned into the *SfiI/NotI*-digested backbones of EXA1 and pEGS3XA1.

To generate plasmids pIIXA1, pIIGS1XA1 and pIIGS3XA1, PCR primers Gal4 IIback and Gal4 IIfor were used to produce PCR fragments by priming off each other and then outer primers Gal4back and Gal4for were used to amplify the fragments further. The PCR products were digested with *SfiI* and *NotI* and cloned into the *SfiI/NotI*-digested backbones of EXA1, pEGS1XA1 and pEGS3XA1. The correct sequence of all constructs was verified by DNA sequencing.

The following oligonucleotides (with restriction sites underlined) were used :

NotGS1XA1back, 5'-GCA AAT CTG CGG CCG CAG GTG GAG GCG GTT CAA TCG AGG GAA GGA TGG CAG AG-3' (Seq ID No. 10);

NotGS3XA1back, 5'-GCA AAT CTG CGG CCG CAG GTG GAG GCG GTT CAG GCG GAG GTG GCT CTG GCG GTG GCG GAT CGA TCG AGG GAA GAA TGG CAG AG-3' (Seq ID No. 11);

Gal4 VLback (containing *SfiI* site), 5'-GGC ATT CAT GCG GCC GCG GCC CAG CCG GCC ATG AAG CAA CTA GAA GAC AAG GTG GAG GAA CTC CTT AGC AAG GTA TAC C-3' (Seq ID No. 12);

Gal4 VLfor (containing *NotI* site), 5'-GCA AAT CTG CGG CCG CCT CTC CAA CAA GCT TCT TCA GTC GAG CGA CTT CGT TCT CAA GAT GGT ATA CCT TGC TAA GGA G-3' (Seq ID No. 13);

Gal4 AAback (containing *SfiI* site), 5'-GGC ATT CAT GCG GCC GCG GCC CAG CCG GCC ATG AAG CAA GCA GAA GAC AAG GCA GAG GAA GCT CTT AGC AAG GCT TAC C-3' (Seq ID No. 14);

Gal4 AAfor (containing *NotI* site), 5'-GCA AAT CTG CGG CCG CCT CTC CAG CAA

GCT TCT TTG CTC GAG CAG CTT CGT TCT CTG CAT GGT AAG CCT TGC TAA GAG C-3' (Seq ID No. 15);

Gal4 IIback (containing *Sfi*I site), 5'-GGC ATT CAT GCG GCC GCG GCC CAG CCG GCC ATG AAG CAA ATC GAA GAC AAG ATA GAG GAA ATT CTT AGC AAG ATC TAC C-3' (Seq ID No. 16);

Gal4 IIfor (containing *Not*I site), 5'-GCA AAT CTG CGG CCG CCT CTC CTA TAA GCT TCT TGA TTC GAG CAA TTT CGT TCT CTA TAT GGT AGA TCT TGC TAA GAA TTT C-3' (Seq ID No. 17);

Gal4 back, 5'-GGC ATT CAT GCG GCC GCG GC-3' (Seq ID No. 18);

Gal4 for, 5'-GCA AAT CTG CGG CCG CCT CTC-3' (Seq ID No. 19); and 4070Afor (described above).

Target cell lines and production of viruses

GP+Env AM12 cells (Markowitz *et al.*, Virology *167*:400-406) were derived from the murine cell line NIH 3T3 and express the MLV-A envelope which blocks the RAM-1 receptor by interference. NIH 3T3, GP+Env AM12 and the human cell line A431 (Giard *et al.*, J. Natl. Cancer Inst. *51*, 1417-1421), were grown in DMEM supplemented with 10% fetal calf serum. The different envelope expression constructs were transfected into TELCeB6 packaging cells (Cosset *et al.*, J. Virol. *69*:7430-7436) by calcium phosphate precipitation (Takeuchi *et al.*, J. Virol. *68*:8001-8007) and stable phleomycin (50mg/ml) resistant colonies were expanded and pooled. Cells were grown in DMEM supplemented with 10% fetal calf serum and when confluent transferred from 37°C to 32°C and incubated for 72hrs. Supernatants containing retroviral particles were harvested after overnight (16hrs) incubation at 32°C in 10mls serum-free DMEM for infections, or DMEM supplemented with 2% fetal calf serum for immunoblots. All supernatants were filtered (0.45μm) before use.

Immunoblots

Virus producer cells were lysed in a 20mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 0.05% SDS, 5mg/ml sodium deoxycholate, 150mM NaCl and 1mM PMSF. Lysates were incubated for 10 mins at 4°C and were centrifuged for 10 mins at 10,000 x g to pellet the nuclei. Virus samples were obtained by ultracentrifugation of filtered viral supernatants (10ml) at 30 000 rpm in a SW40 rotor (Beckman, USA) for 1 hr at 4°C. The pelleted viral particles were resuspended in 100μl PBS. Samples (30μl for cell lysates, or 10μl for pelleted virions) were then separated on a 10% polyacrylamide gel under reducing conditions followed by transfer of the proteins onto nitrocellulose paper. For Factor Xa cleavage, 10μl of the pelleted viral particles were incubated with 0 or 4 μg/ml of Factor Xa (Promega, USA) for 90 min at 37°C in the presence of 2.5mM CaCl, before running on the separating gel. The SU proteins were detected as previously described (Cosset *et al.*, J. Virol. *69*:6314-6322) using specific goat antibodies raised against either Rausher leukaemia virus (RLV) gp70 SU or RLV p30 capsid protein (CA) (Quality Biotech Inc, USA) which were diluted 1/1,000 and 1/10,000 respectively. Blots were developed with horseradish peroxidase-conjugated rabbit anti-goat antibodies (DAKO, Denmark) and an enhanced chemiluminescence kit (Amersham Life Science, UK).

Target cell Infection

Target cells were seeded at 2 x 10^5 cells/well in six-well plates and incubated at 37°C overnight. Producer cell supernatants containing β-galactosidase-transducing retroviruses were filtered (0.45μm) after overnight incubation at 32°C in serum-free medium. The harvested supernatants were incubated with 0 or 4 μg/ml of factor Xa (Promega) for 90 minutes at 37°C in the presence of 2.5mM $CaCl_2$. Supernatant dilutions in 2ml serum-free media were incubated with target cells for 6 hrs in the presence of 8μg/ml polybrene. The retroviral supernatant was then removed and the cells were incubated with regular medium for 48-72 hrs. X-Gal staining for detection of β-galactosidase activity was performed as previously described (Tatu *et al.*, EMBO J. *14*:1340-1348). Viral titre (enzyme forming units/ml) was calculated by counting blue stained colonies microscopically with the use of a grid placed underneath the 6 well plates.

RESULTS

Rec-1 mediated infection by envelopes expressing a Ram-1 targeting domain

AMo1 and AXMo1 are previously described chimaeric envelopes in which the RAM-1 receptor binding domain from 4070A SU is fused to aminoacid +1 of Moloney SU by a noncleavable (AAA) or factor Xa-cleavable (AAAIEGR) linker (Nilson *et al.*, Gene Ther. *3*:280-286). Viruses incorporating the AMo1 and AXMo1 envelopes were pelleted, cleaved with 0 or 4μg/ml factor Xa protease and then analysed on immunoblots using an anti-envelope antiserum as a probe.

The reversible inhibition of infection by retroviral incorporation and cleavage of chimaeric envelopes expressing a factor Xa-cleavable, N-terminal RAM-1 binding domain is shown in Figure 6A and 6B. Figure 6A is an immunoblot of pelleted recombinant retroviral particles incorporating Mo, AMo1 or AXMo1 envelopes before (-) or after (+) treatment with factor Xa protease, probed with antiserum to the SU glycoprotein. Figure 6B shows the results when the target cell line GP+Env AM12 was infected with harvested producer cell supernatants containing β-galactosidase-transducing retroviruses (AMo1, AXMo1, Mo and A) with or without treatment with factor Xa protease. Detection of β-galactosidase activity was performed by X-gal staining and titres were expressed as e.f.u./ml.

It is apparent from Figure 6A that the chimaeric envelopes were incorporated into virions with equal efficiency (although less efficiently than the unmodified Moloney SU) and that AXMo1, but not AMo1 envelopes, were cleaved by factor Xa protease to yield an SU cleavage product whose mobility was indistinguishable from unmodified Mo SU.

The infectivity of these Ram-1 targeted vectors was then tested on NIH3T3 cells and on NIH3T3 transfectants (GP+Env AM12) overexpressing the 4070A envelope which blocks the corresponding Ram-1 receptor by interference. The vectors AMo1 and AXMo1 were fully infectious on the unmodified NIH3T3 cells which express both Rec-1 and Ram-1, giving titres in excess of 10^6 efu per ml (not shown), however their infectivity was greatly reduced on the Ram-1 deficient cells, suggesting that they were unable to utilise the ecotropic receptor, Rec-1 (Fig. 6B). This result was unexpected and was in contrast to results obtained with similar chimaeric Moloney SU glycoproteins displaying monomeric growth factor domains or single chain antibody fragments in which Rec-1 mediated

infection was not seriously compromised by the displayed domains (Ager *et al.*, Human Gene Ther., in press; Cosset *et al.*, J. Virol. *69*:6314-6322). This led to the proposal that the displayed Ram-1 binding domain might be forming a trimeric complex at the tip of the Moloney SU glycoprotein trimer to which it was grafted, thereby blocking the Rec-1 binding site and/or interfering with Rec-1 mediated fusion triggering. Such a block would be expected to be reversible by cleaving the Ram-1 binding domain from the vector and, in keeping with this prediction, the infectivity of the AXMo1 vector was fully restored on Rec-1 positive, Ram-1 deficient cells when the Ram-1 targeting domain was cleaved from its surface with factor Xa protease (Fig. 6B).

Construction of chimaeric 4070A envelopes displaying helical peptides

To further test the idea that a trimeric polypeptide could block the functions of a trimeric envelope glycoprotein when fused to its N-terminus, and to determine whether the concept could be applied to an amphotropic MLV SU glycoprotein, we made a series of constructs coding for chimaeric envelopes in which monomeric or trimerising helical peptides were fused to amino acid +1 of 4070A SU via Factor Xa-cleavable linkers (Fig. 7). The helical peptides that were chosen for these studies were variants of the dimeric GCN4 leucine zipper peptide with systematic V, L, I or A (single letter aminoacid code) substitutions in the **a** and **d** positions of the heptad repeat that are known to force the formation of trimeric coiled coils (VL and II peptides) or to prevent oligomerisation (AA peptide) (Harbury, *et al.*, Science *262*:1401-1407). When designing these constructs, we were concerned that the oligomerisation of the displayed VL and II peptides might be hindered if they were tethered too closely to the underlying 4070A SU glycoprotein. The spacing between the 4070A SU glycoprotein and the displayed peptide motifs was therefore varied by insertion of linkers comprising amino acids AAA**IEGR**, Seq ID No. 20), AAAGGGGS**IEGR** (Seq ID No. 8) or AAAGGGGSGGGGSGGGGS**IEGR** (Seq ID No. 9), where the highlighted sequence is known to be recognised and cleaved by Factor Xa (Nilson *et al.*, Gene Ther. *3*:280-286).

Expression, viral incorporation and cleavage of chimaeric 4070A envelopes

The AA, VL and II chimaeric envelopes and a control amphotropic (4070A) envelope were stably transfected into TELCeB6 cells which express MLV gag-pol core particles and

an nls LacZ retroviral vector (Cosset *et al.*, J. Virol. *69*:7430-7436). Virus-containing supernatants were harvested from these stably transfected TELCeB6 cells and ultracentrifuged to pellet the viral particles. Pellets were than analysed on immunoblots for the presence of viral core proteins and envelope proteins (Fig. 8A).

Figure 8 illustrates the viral incorporation and cleavage of chimaeric envelopes expressing factor Xa-cleavable helical peptides as N-terminal extensions of the 4070A MLV SU. Figure 8A is an immunoblot of pelleted retroviral particles incorporating chimaeric envelopes. The lane contents are as follows: 1:VLXA1, 2:VLGS1XA1, 3:VLGS3XA1, 4:AAXA1, 5:AAGS3XA1, 6:IIXA1, 7:IIGS1XA1, 8:IIGS3XA1, and 9:A. The top immunoblot was probed with an anti-SU antiserum and the lower one with an anti-p30 antiserum to detect the p30 CA protein.

Figure 8B shows the Factor Xa-mediated cleavage of chimaeric envelopes and takes the form of an immunoblot of pelleted recombinant amphotropic retroviral particles incorporating A, VLXA1, AAXA1, IIXA1 or EXA1 envelopes before (-) or after (+) treatment with factor Xa protease, probed with anti-SU antiserum.

Figure 8C is an immunoblot of cell lysates prepared from the virus producing TELCeB6 transfectants A, VLXA1, AAXA1, IIXA1 and the control, untransfected TELCeB6, probed with anti-SU antiserum.

The number of vector particles present in each sample, determined by staining with p30 antiserum to detect the p30 CA protein, was found to be approximately equivalent (Fig. 8A). However, when the efficiencies of viral incorporation of the different chimaeric envelopes were compared, by staining with an anti-SU antiserum, it was found that incorporation is greatly influenced by the presence of the oligomerizing peptide. Envelopes displaying the control monomeric peptide (AA) were incorporated almost as efficiently as wild type 4070A envelopes whereas envelopes displaying the VL peptide were incorporated much less efficiently and there was no visible incorporation of envelopes displaying the II peptide. To determine if the helical peptides could be cleaved from the SU glycoproteins to which they were grafted, viral pellets were digested with 0

or 4 μg/ml factor Xa protease and then analysed on immunoblots as before.

Figure 8B shows that there is a mobility shift when expressed envelopes VLXA1, AAXA1 and the control EXA1, have been cleaved with factor Xa protease, indicating that the helical peptides are indeed cleaved from the SU. Due to the low levels of incorporation of the IIXA1 chimaeric envelope, cleavage can not be seen for this vector. This immunoblot also indicates that the chimaeric envelope AAXA1 was incorporated 10 times more efficiently than VLXA1.

To further investigate the poor incorporation of the VL and II chimaeric envelopes we performed immunoblots of cell lysates prepared from the virus producing TELCeB6 transfectants. Figure 8C shows that the unprocessed precursors of all three chimaeric envelopes are detectable in the cell lysates. However, the VL and II envelope precursors are less abundant than the AA precursor. Also, the processing of the VL and II precursors to mature SU is severely impaired relative to the processing of the AA precursor indicating that these chimaeric envelopes are not efficiently transported from the endoplasmic reticulum to the Golgi compartment.

Infectivity of vectors displaying chimaeric envelopes before and after cleavage

To determine whether the helical peptides were masking the functions of the 4070A envelopes to which they were fused we titrated the vectors on Ram-1 expressing cells, NIH3T3 and A431 before and after they were cleaved with factor Xa protease (Table 4 and Figure 9). Figure 9 shows the reversible inhibition of infection by cleavage of the chimaeric envelope, VLXA1, expressing a factor Xa-cleavable, N-terminal oligomerizing peptide and is a magnified view of virally infected cells after X-gal staining. Chimaeric envelope VLXA1 shows strong inhibition of infectivity on NIH 3T3 and A431 cells, which is reversible on addition of factor Xa.

The control vectors displaying the AA peptide gave titres comparable to that of the wild type amphotropic vector and the titres did not change after factor Xa cleavage indicating that the AA peptide does not significantly interfere with the functions of the underlying 4070A envelope. Conversely, the vectors displaying the trimerising VL and II helical

peptides gave greatly reduced titres on both cell lines which were enhanced as much as 2000-fold by factor Xa cleavage. In the case of the VLXA1 chimaeric envelope, on cleavage with 4 μg/ml factor Xa protease, the titre on NIH3T3 cells increased from 151 efu/ml to 3 x 10^5 efu/ml and on A431 cells from 318 efu/ml to 10^5 efu/ml (Fig. 9). Vectors displaying the II helical peptide gave generally lower titres than those displaying the VL peptide, presumably due to the reduced incorporation of chimaeric envelopes displaying the II peptide. Interdomain spacing had little apparent influence on the titre of the uncleaved vectors, nor on the degree of titre enhancement that was observed after exposure to the factor Xa protease.

Titre (e.f.u./ml) of harvested β-galactosidase-transducing retroviruses																		
	A		AAXA1		AAGS3XA1		VLXA1		VLGS1XA1		VLGS3XA1		IIXA1		IIGS1XA1		IIGS3XA1	
Factor Xa[a]	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+
NIH3T3	10^7	10^7	$2x10^6$	$2x10^6$	$2x10^6$	$2x10^6$	151	$3x10^5$	$5x10^3$	$2x10^4$	$2x10^4$	$3x10^5$	6	10^3	34	10^3	10^3	$8x10^3$
A431	10^7	10^7	$2x10^6$	$2x10^6$	$2x10^6$	$2x10^6$	318	10^5	10^3	$5x10^4$	10^3	$3x10^5$	10	10^3	16	$6x10^2$	162	10^4

[a] Harvested producer cell supernatants containing β-galactosidase-transducing retroviruses were preincubated with (-) or without (+) factor Xa protease.

Reversible inhibition of infection by cleavage of chimaeric envelopes expressing a factor Xa-cleavable, N-terminal oligomerizing peptide.

TABLE 4.

DISCUSSION

In the above example we have shown that the Ram-1 binding domain from the homotrimeric 4070A SU glycoprotein can inhibit Rec-1 mediated infection by the homotrimeric Moloney SU glycoprotein when grafted to its N-terminus. We have also shown that short trimeric leucine zipper peptides, but not a monomeric helical peptide, can inhibit Ram-1 mediated infection by the 4070A envelope when fused to its N-terminus. In both cases, by using factor Xa protease to cleave the trimeric N-terminal extensions from the virally incorporated envelopes, it was possible to reverse the block to Rec-1 or Ram-1 mediated infection. We propose that the masking of envelope functions by these inhibitory N-terminal extensions is a consequence of their assembly into a trimeric complex at the tip of the SU glycoprotein trimer to which they are grafted.

The VL, II and AA peptides that we fused to the 4070A envelope are mutants of the GCN4 leucine zipper in which the conserved, buried residues that direct dimer formation have been substituted with valine, leucine, isoleucine or alanine residues (Harbury *et al.*, Science *262*:1401-1407). The VL mutant oligomerises to form extremely stable (T_m 95°C) two- and three-stranded alpha-helical coiled coil structures whereas the II mutant forms exclusively three-stranded coiled coils which are even more stable ($T_m > 100$°C) than the VL structures. In the AA peptide, all of the hydrophobic core residues of the GCN4 leucine zipper were substituted with alanines to prevent oligomerisation of the mutant peptide whilst preserving its helical structure.

Retroviral incorporation of chimaeric envelopes displaying the VL and II peptides was significantly impaired relative to chimaeric envelopes displaying the control AA peptide, which showed only a slight reduction in incorporation compared to unmodified 4070A envelopes. The VL chimaeric envelopes were approximately ten-fold less abundant in viral pellets than the AA chimaeric envelopes, and the II chimaeric envelopes were so poorly incorporated that they were not visible on immunoblots of pelleted virions. By immunoblotting cell lysates from the transfected TELCeB6 cells with anti-envelope antiserum, it was shown that the intracellular abundance of the precursor polypeptides for each of the chimaeric envelopes was closely correlated with their abundance in viral pellets. The low viral incorporation of the VL and II chimaeric envelopes is therefore a

consequence of their poor expression and/or folding in the virus producing cells.

It is currently unclear what is responsible for the impaired expression of the VL and II chimaeric envelopes. Neither protein appears to be toxic to the virus producing cells since there was no difference in the number or size of stably transduced TELCeB6 clones that were obtained after transfecting the different (oligomerising or control) chimaeric envelope expression plasmids (data not shown). An alternative possibility might be that the low intracellular abundance of the VL and II envelope precursors is due to their premature oligomerisation in the endoplasmic reticulum. Premature oligomerisation of the nascent polypeptide chains via their N-terminal VL or II peptides might seriously compromise the folding of individual subunits leading to their aggregation and accelerated proteolytic destruction. In keeping with this idea, in a related system the chaperone-guided folding of influenza haemagglutinin monomers is known to be completed in the endoplasmic reticulum before the fully folded subunits can be assembled into homotrimers (Valsesia-Wittmann *et al.*, J. Virol. *68*:4609-4619). The fact that the II helical peptide forms very stable trimers and that the VL peptide forms slightly weaker interactions might then explain why the chimaeric envelopes displaying the VL peptides gave better incorporation than the chimaeric envelopes displaying the II peptides. To test this idea, we are planning to generate chimaeric envelopes displaying trimeric leucine zipper peptides with reduced stability (i.e. lower melting temperatures) compared to the VL and II peptides that were used in this study.

All vectors carrying the VL or II chimaeric envelopes showed inhibition of infection on NIH3T3 and A431 cells, which was reversible on cleaving the peptides from the vectors with factor Xa protease. Titres were not restored completely to wild type levels due to the reduced levels of incorporation of these envelopes. The VL and II peptides therefore function as oligomerising peptide adaptors which mask the functions of the retroviral envelope glycoprotein to which they are fused. The inhibition of infection may be as a result of the oligomerizing peptide blocking binding of the vector to its target cells by masking the underlying binding domain. Alternatively, the presence of an oligomerizing peptide may prevent dissociation of the envelope trimer, blocking fusion. Unfortunately, because of the impaired incorporation of the VL and II chimaeric envelopes, binding

studies were uninformative so we are unable to determine which of these mechanisms is more dominant.

A low level of background infectivity was consistently observed when uncleaved vectors displaying the VL and II peptides were used to infect NIH3T3 and A431 cells (Table 4). The background was slightly higher on the NIH3T3 cells than on the A431 cells and tended to increase with increasing length of the linker peptide that was inserted between the 4070A SU and the oligomerizing peptides. We believe that this background infectivity occurs because a few of the chimaeric envelopes are cleaved by endogenous proteases derived from the target cells. In previous studies using chimaeric envelopes displaying a cleavable EGF domain we have observed that the IEGR factor Xa cleavage site can be cleaved to a small extent by proteases released from NIH3T3 and A431 cells. We also found that increasing the length of the linker sequence between the factor Xa cleavage site and the displayed EGF domain increased the accessibility of the factor Xa site to these endogenous proteases.

In summary, our results demonstrate that retroviral vector infectivity can be reversibly inhibited by fusing cleavable trimeric peptide adaptors to the N-terminus of the 4070A SU envelope glycoprotein. Infectivity is restored by exposing the vector particles to a protease that cleaves the adaptor from the SU glycoprotein. It is anticipated that these adaptors will be useful to prevent infection of nontarget cells in the two-step (targeted attachment, targeted cleavage) targeting strategies that we are currently developing. Chimaeric envelopes displaying the VL and II peptide adaptors that were used in this study were poorly expressed. We are therefore attempting to identify similar masking adaptors that do not compromise the expression of chimaeric envelopes on which they are displayed.

Example 4

Construction of retroviruses containing cleavable oligomerising adaptors with an EGF binding domain

MATERIALS AND METHODS

Plasmid Construction

Vectors pEGF LVA1 and pEGF LVXA1 display an oligomerising peptide, LV, fused to residue +1 of 4070A SU with a non cleavable (SAA) or factor Xa protease-cleavable (SAAIEGR, Seq ID No. 21) linker and also display the EGF binding domain. To generate these vectors, PCR primers Gal4 LV, Gal4 LVbak and Gal4 LVfor were used for assembly of the PCR fragment coding for the oligomerising peptide, LV (Harbury *et al.*, 1993 Science *262*, 1401-1407). The PCR product was digested with *NotI* and *EagI* and cloned into the *NotI*-digested backbones of EA1 and EXA1. Figure 10 shows a diagramatic representation of the two constructs. The correct sequence of the constructs was verified by DNA sequencing.

+The following oligonucleotides (with restriction sites underlined) were used:

Gal4 LV, 5'-GAC AAG CTA GAG GAA GTA CTT AGC AAG CTC TAC CAT GTC GAG AAC GAA CTT GCT CGA GTT AAG AAG-3' (Seq ID No. 22);

Gal4 LVback (containing *Not*I site), 5'-GGC ATT CAT GCG GCC GCA ATG AAG CAA GTG GAA GAC AAG CTA GAG GAA GTA C-3' (Seq ID No. 23);

Gal4 LVfor (containing *Eag*I site), 5'-GCA AAT CTG CGG CCG ACT CTC CCA GAA GCT TCT TAA CTC GAG CAA GTT C-3' (Seq ID No. 24).

Target cell lines and production of viruses

The murine cell line NIH 3T3, and the human cell line A431, were grown in DMEM supplemented with 10% fetal calf serum. The envelope expression constructs were transfected into TELCeB6 packaging cells by calcium phosphate precipitation and stable phleomycin (50mg/ml) resistant colonies were expanded and pooled. Cells were grown in DMEM supplemented with 10% fetal calf serum and when confluent transferred from 37°C to 32°C and incubated for 72hrs. Supernatants containing retroviral particles were harvested after overnight (16hrs) incubation at 32°C in 10mls serum-free DMEM for infections. All supernatants were filtered (0.45μm) before use.

Target cell Infection

Target cells were seeded at 2 x 10^5 cells/well in six-well plates and incubated at 37°C overnight. The harvested supernatants containing β-galactosidase-transducing retroviruses were incubated with 0 or 4 μg/ml of factor Xa (Promega) for 90 minutes at 37°C in the presence of 2.5mM $CaCl_2$. Supernatant dilutions in 2ml serum-free media were incubated with target cells for 6 hrs in the presence of 8μg/ml polybrene. The retroviral supernatant was then removed and the cells were incubated with regular medium for 48-72 hrs. X-Gal staining for detection of β-galactosidase activity was performed and viral titre (enzyme forming units/ml) was calculated by counting blue stained colonies microscopically with the use of a grid placed underneath the 6 well plates.

Host range properties of virus incorporating chimeric envelopes

To determine whether the oligomerising peptide was masking the functions of the 4070A envelope to which it was fused, we titrated the vectors on Ram-1 expressing cells, NIH3T3 and A431 before and after they were cleaved with factor Xa protease (Fig. 11). Both vectors gave greatly reduced titres on NIH3T3 and A431 cells compared to wildtype amphotropic vector. On cleavage of EGF LVXA1 with 4 μg/ml factor Xa protease the titre on NIH3T3 cells and A431 cells increased by up to 200 fold. Cleavage of the contol vector EGF LVA1, which does not carry the factor Xa cleavage signal, however, did not result in such an increase in titre.

These data demonstrate that a monomeric binding domain can be displayed as part of a trimerising adaptor which blocks the function of the underlying envelope until it is cleaved with a specific protease.

Example 5

Angiogenesis, inflammation and tumour invasion are linked to the overexpression of matrix metalloproteinases (MMPs) which degrade the extracellular matrix. The MMPs are therefore promising targets for therapy. As an alternative to using MMP inhibitors, we are developing MMP-activatable gene delivery systems. Here, we describe the construction of vectors incorporating inhibitory adaptors that are efficiently cleaved by activated MMPs. The MMP-sensitive vectors underwent cleavage activation selectively

on target cells expressing endogenous membrane-associated MMPs, and gene delivery was dramatically enhanced. MMP-activatable vectors will offer new opportunities for targeting of therapeutic genes to sites of disease.

Matrix metalloproteinases (MMPs) are important for angiogenesis, tissue remodelling, inflammation and wound healing, and they play a crucial role in various pathological processes including cancer invasion and metastasis and the destruction of articular cartilage in rheumatoid arthritis (Liotta *et al.*, 1991 Cell *64*, 327; Woessner Jr., 1991 FASEB J. *5*, 2145; Ray & Stetler-Stevenson 1994 Eur. Respir. J. *7*, 2062; Karelina *et al.*, 1995 J. Invest. Dermatol. *105*, 411). The known MMPs include matrilysin, collagenases1-3, stromelysins1-3, gelatinases A and B and a group of 4 membrane-type MMPs (MT-MMP) which are anchored to cell membranes (Sato *et al.*, 1994 Nature *370*, 61; Takino *et al.*, 1995 J. Biol. Chem. *270*, 23013; Will & Hinzmann 1995 Eur. J. Biochem. *231*, 602; Puente *et al.*, 1996 Can. Res. *56*, 944). Most of the MMPs are secreted as zymogen forms and require activation before they can exert their proteolytic activities. The net activities of the enzymes are also regulated by the three tissue inhibitors of MMPs (TIMPs 1-3). Once activated, the MMPs co-operate with one another in a cascade pathway to cause degradation of the extracellular matrix. Gelatinase A (GLA; MMP-2) and the MT-MMPs are of special interest with respect to tumour invasion. Pro-GLA is secreted by stromal fibroblasts and concentrated on tumour cell membranes, especially at the invasive front of the tumour (Afzal *et al.*, 1996 Lab. Invest. *74*, 406; Nomura *et al.*, 1996 Int. J. Can. (Pred. Oncol.) *69*, 9). It binds as a pro-GLA-TIMP-2 complex to MT1-MMP which then mediates its cleavage activation on the surface of the tumour cell (Strongin *et al.*, 1995 J. Biol. Chem. *270*, 5331; Emmert-Buck *et al.*, 1995 FEBS Letters *364*, 28; Gilles *et al.*, 1996 Int. J. Can. *65*, 209). Indeed, MT1-MMP-mediated activation of pro-GLA is considered to be important for the progression of cancer and the concentration of active GLA is often found to be elevated in invasive or metastatic tumours. Hence, there is considerable interest in the exploitation of MMPs as promising targets for novel therapeutic agents and there are several general or specific MMP-inhibitors that are currently being tested for their usefulness in treatment of MMP-linked diseases in a number of clinical trials (Hodgson 1995 Biotech. *13*, 554; Eccles *et al.*, 1996 Can. Res. *56*, 2815). Here, as an alternative to the use of MMP-inhibitors, we propose the use of

a MMP-activatable gene delivery system.

This example describes the generation of targeted retroviral vectors whose infectivity for human EGF receptor-expressing cancer cells is strongly activated by membrane-associated MMPs.

A series of chimaeric envelope expression constructs was generated in which a cDNA coding for the 53 amino acid receptor binding domain of EGF was linked to the N-terminal codon of the 4070A murine leukemia virus (MLV) SU envelope glycoprotein via short non-cleavable or protease-cleavable linkers. In brief, the chimaeric vectors E.A and E.X.A, have an EGF cDNA, flanked by *Sfi*I and *Not*I restriction sites, inserted at codon +1 of the N-terminus of wild type 4070A MLV SU (surface protein gp 70) envelope, with a linker of either 3 alanines (E.A.) or 3 alanines and the IEGR Factor Xa cleavage sequence (E.X.A.) between the domains. Figure 12 is a schematic representation of the chimaeric envelope expression constructs, E.A, E.G_4S.A, E.X.A and E.MMP.A. The envelope constructs were transfected into TELCeB6 complementing cells, virus-producing clones were pooled and expanded in 10% FCS-DMEM selection medium containing 50 μg/ml phleomycin. Arrows indicate potential site of cleavage by respective proteases.

To obtain constructs E.MMP.A and E.G_4S.A, PCR primers A1GelA Nb (5' GCA AAT CTG CGG CCG CAC CTT TGG GAC TTT GGG CAA TGG CAG AGA GCC CCC ATC, Seq ID No. 27) or NL1A1B (5' GCA AAT CTG CGG CCG CAG GTG GAG GCG GTT CAA TGG CAG AGA GCC CCC ATC, Seq ID No. 28) respectively, were used with primer 4070Afor (described above) on E.A to generate *Not*I-tailed PCR fragments of the 4070A SU coding sequence encoding the MMP-cleavable (PLGLWA) or non-cleavable linker (G_4S) as a 5' extension. The PCR fragments were digested with *Not*I and *Bam*HI and cloned into the *Not*I-*Bam*HI digested backbone of E.A to generate constructs E.MMP.A and E.G_4S.A. The sequences of the constructs were checked and verified by DNA sequencing.

The E.A and E.G_4S.A chimaeric envelopes contained non-cleavable linkers AAA and AAAGGGGS (Seq ID No. 25) respectively (single letter amino acid code), the E.X.A

envelope contained a Factor Xa-cleavable linker AAAIEGR and the E.MMP.A envelope contained the linker AAAPLGLWA (Seq ID No. 26) in which the highlighted sequence is known to be recognised and cleaved by GLA and by MT1-MMP (Ye *et al.*, 1995 Biochem. *34*, 4702; Will *et al.*, J. Biol. Chem. *271*, in press) (Figure 12).

The chimaeric envelope constructs and a wild type 4070A envelope expression construct were stably transfected into TELCeB6 complementing cells which express Moloney MLV gag-pol proteins and the nlsLacZ retroviral vector, as described in the preceding examples. Upon transfection of these cells with a functional envelope expression plasmid, infectious enveloped vector particles capable of transferring the lacZ marker gene are rescued into the culture supernatant. Viral supernatants were harvested from confluent plates of pooled transfected TELCeB6 cells and the viral particles were pelleted by ultracentrifugation and immunoblotted using an anti-envelope antiserum as probe. Immunoblotting was performed as described in the preceding examples. The results are shown in Figure 13A, B.

Figure 13A is an immunoblot showing comparative viral incorporation of the EGF chimaeric vectors (lane 2=E.A; lane 3=E.X.A; lane 4=E.MMP.A; lane 5=E.G_4S.A) and the wild type 4070A SU (lanes 1 and 6). Figure 13B is an immunoblot demonstrating cleavage of MMP-cleavable linker in E.MMP.A by purified p-aminophenylmercuric acetate (APMA)-activated gelatinase A (GLA). An aliquot of the E.MMP.A viral pellet was incubated, respectively, with PBS only (lane 1), APMA-activated GLA (final concentration 32 μg/ml; lane 2) and APMA at a final concentration of 2 mM (lane 3). Lane 4 shows unmodified wild type 4070A-SU.

It is apparent from Figure 13A that all four chimaeric envelopes were expressed and incorporated into virions, as indicated by the decrease in mobility compared to wild type 4070A-SU, and that the relative efficiencies of envelope incorporation were comparable in the four different recombinant virus stocks.

To determine if GLA could recognise the PLGLWA sequence on the E.MMP.A vector and thus cleave the EGF domain from the chimaeric viral envelope without degrading the underlying 4070A SU glycoprotein, we incubated aliquots of the E.MMP.A and control

viral pellets for 30 min at 37°C with PBS, p-aminophenylmercuric acetate (APMA) or APMA-activated GLA, after which immunoblots were performed as before. [Gelatinase A (GLA) was purified as a zymogen form and requires activation by incubation with APMA (2 mM) for 1 h at 25°C prior to use. 10μl of the resuspended E.X.A, E.G$_4$S.A or E.MMP.A viral pellets were incubated with PBS, APMA (final concentration 2 mM) or APMA-activated GLA (32 μg/ml) for 30 min at 37°C].

On treatment of E.MMP.A-SU with activated GLA, a band with the same mobility as the wild type 4070A-SU was recovered, indicating that the EGF domain could be efficiently cleaved from this chimaeric envelope without further GLA-mediated degradation (Fig. 13B). The E.G$_4$S.A and E.X.A chimaeric envelopes were unaffected by treatment with GLA indicating that cleavage was specific for the MMP-sensitive linker (not shown).

Our previous data (Nilson *et al.*, 1996 Gene Therapy *3*, 280) indicated that the infectivity of the E.X.A vector was minimal on EGF-receptor positive A431 cells but could be fully and selectively restored by cleaving the chimaeric envelope with Factor Xa protease. This result was confirmed using the E.X.A vector stocks that were generated in the current study which bound strongly to EGF receptors on A431 cells and gave a titre of 10^3 efu/ml rising to 10^6 efu/ml after the EGF domain was cleaved from their surface with Factor Xa protease (data not shown). The infectivities of the E.A, E.G$_4$S.A and E.MMP.A vectors on A431 cells were low between 10^2-10^3 efu/ml and were not greatly increased by treatment with Factor Xa protease (not shown).

To determine whether the infectivity of the MMP-cleavable E.MMP.A vector could be activated by GLA, we performed infections on A431 cells in the presence of increasing concentrations of exogenous pro-GLA. Since A431 cells are known to activate pro-GLA to GLA, pre-activation of the protease with APMA was not necessary.

For the infection assays, A431 cells in 10% FCS-DMEM were seeded, at a density of 3 x 10^4 per individual well, in a 24-well tissue culture plate (Corning, New York) overnight at 37°C. The media were removed the next day and the cells were washed once in serum-free DMEM. Varying amounts (final concentration 2-40 μg/ml) of pro-GLA were mixed

with 200 μl of filtered E.MMP.A viral supernatant after which the mixture was added to A431 cells and incubated at 37°C for 6 h. At the end of 6 h, the media was removed and cells were washed once in serum-free DMEM. The cells were then incubated in 10% FCS-DMEM for 72 h at 37°C before they were washed once in cold PBS, fixed in 0.5% gutaldehyde-PBS for 15 min, washed once again with PBS and incubated with X-gal overnight at 37°C. The number of colonies transduced with the vector (blue colonies) were counted and the titre expressed as efu/ml viral supernantant. Figure 14 is a graph showing that increase in titre (efu x 10^{-4}/ml) of the E.MMP.A MMP-sensitive vector on A431 cells is correlated with the amount of pro-gelatinase A (pro-GLA) added onto the cells.

It was found that as the concentration of exogenous pro-GLA was increased incrementally from 2 to 40 μg/ml, the infectivity of the E.MMP.A vector increased in a dose-dependent manner. From 1.3 x 10^3 efu/ml in the absence of pro-GLA, the titre increased 50-fold to 6.5 x 10^4 efu/ml in the presence of 40 μg/ml pro-GLA. The titre of the noncleavable E.G_4S.A vector was relatively unchanged from 1.2 x 10^2 efu/ml in the absence of pro-GLA to 1.4 x 10^2 efu/ml in the presence of 40 μg/ml pro-GLA. Activation of infectivity was specific to the vector with the MMP-cleavable linker as infectivity of E.X.A increased only 3-fold in the presence of 40 μg/ml pro-GLA (not shown).

We next explored the possibility that endogenous target cell-derived MMPs could activate the E.MMP.A vector in the absence of exogenous MMP. HT1080 is a human fibrosarcoma cell line that constitutively produces MT1-MMP and pro-GLA (Okada *et al.*, 1995 Proc. Natl. Acad. Sci. *92*, 2730). We therefore incubated the viral supernatants of E.G_4S.A, E.X.A and E.MMP.A on HT1080 and A431 cells for 6 h at 37°C in the absence of exogenously added pro-GLA: 200μl of the filtered E.X.A, E.G_4S.A or E.MMP.A viral supernatants were added onto A431 or HT1080 cells for 6 h at 37°C with 8 μg/ml polybrene, after which the incubation medium was removed and the cells washed once in serum-free DMEM. The cells were incubated in 10% FCS-DMEM for 72 h at 37°C before they were stained with X-gal.

Figure 15 is a graph showing the titre of EGF chimaeric vectors on A431 and HT1080

cells. Figure 15A shows the high infectivity of E.MMP.A vector on HT 1080 cells compared to on A431 cells as indicated by the number of blue β-galactosidase positive colonies. One ml out of 10 ml filtered E.MMP.A viral supernatant was incubated with 5 mM $CaCl_2$ for 30 min at 37°C before it was incubated on the respective cell types for 6 h at 37°C. At the end of 6 h, the cells were washed in serum-free DMEM and incubated in 10% FCS-DMEM for 72 h after which they were stained with X-gal. The respective titres are expressed as efu/ml viral supernatant.

Consistent with previous results, the infectivity of the vectors on A431 cells was low in the absence of exogenous pro-GLA. However, on HT1080 cells, the infectivity of the MMP-cleavable vector E.MMP.A was activated by two orders of magnitude compared to the MMP-resistant control vectors E.G_4S.A and E.X.A (Fig. 15, 15A). Thus, in the absence of any added exogenous MMP, the higher titre of the MMP-dependent E.MMP.A vector must be due to its cleavage by MMPs produced endogenously by HT1080 cells.

To determine if the MMP-activatable E.MMP.A vector could selectively target the MMP-expressing HT1080 cells in preference over A431 cells, we allowed the vector to infect both cell types on the same petri dish simultaneously. We grew A431 and HT1080 cells separately on coverslips, placed them in the same petri dish and added supernatant containing the E.MMP.A or control vectors: A431 and HT1080 cells were seeded separately, on 25 mm Thermanox coverslips (Corning) contained in 6 well plates, in 10% FCS-DMEM overnight after which the media was removed and the cells washed once in serum-free DMEM. The coverslips coated with the cells were placed in a 10 cm petri dish (Falcon) and E.G_4S.A (1:1.5 dilution), E.MMP.A (1:1.5) or 4070A (1:20) supernatants were added onto the petri dishes with 8 μg/ml polybrene for 6 h at 37°C. At the end of the incubation period, the media was removed and the cells were incubated in 10% FCS-DMEM for 72 h before X-gal staining. The results are shown in Figure 16: E.MMP.A vector grown on HT1080 (H) cells and A431 (A) cells is shown in I and II, with the control E.G_4S.A vector in III and the wild type 4070A vector in IV.

When presented with both cell types, E.MMP.A infected HT1080 cells preferentially over A431 cells. The wild type 4070 A vector and E.G_4S.A vector with the non-cleavable

linker showed no such preference (Fig. 16). In these experiments, the MMP-activatable E.MMP.A vector did not infect A431 cells more efficiently in the presence of HT1080 cells than in their absence. This suggests that soluble GLA released into the medium from the HT1080 cells does not play a significant role in activation of the vector. Instead, the results strongly indicate that the cleavage activation of the E.MMP.A vector is localised to the surface of the HT1080 cells and that it is mediated by membrane-associated MMPs acting on vector particles that have bound to the EGF receptors on these cells.

The significant role that MT-MMP plays in cleavage activation of the MMP-cleavable vector was supported by results from experiments using natural MMP inhibitors TIMP-1 and TIMP-2 and a synthetic inhibitor, CT 1339. For the inhibition studies, TIMP-1 at a final concentration of 10 μg/ml, TIMP-2 (5 μg/ml) or CT 1339 (1 mM) was used. The inhibitors were added to 200 μl of diluted (1:10) E.MMP.A or undiluted E.G_4S.A viral supernatants. The mixture was then added onto A431 or HT1080 cells, which had been washed once in serum free DMEM, and the cells were incubated for 6 h at 37°C. At the end of the incubation period, the cells were washed once in serum free DMEM, incubated for 72 h in 10% FCS-DMEM after which they were stained with X-gal. The E.MMP.A supernatant was diluted to obtain a titre that would allow accurate counting of the number of transduced colonies. Inhibition studies on A431 cells were performed with 200 μl undiluted E.MMP.A or E.G_4S.A in presence of 16 μg/ml pro-GLA.

Table 5 : Influence of MMP inhibitors on the titre of vectors on A431 and HT1080 cells.

Cell type	TIMP-1	TIMP-2	CT 1339
HT 1080	16.7 +/- 2.1	72.7 +/- 9.1	73.3 +/- 6.4
A431	79.3 +/- 10.2	83.3 +/- 8.4	83.4 +/- 9.8

MMP-dependent E.MMP.A vector was added to A431 cells in presence of 16 μg/ml pro-GLA or to HT1080 cells in the absence of exogenous pro-GLA, with or without the addition of natural MMP inhibitors TIMP-1, TIMP-2 or synthetic inhibitor, CT 1339. Values (means $\pm$ SD, n=3) represent percentage decrease in titre (with inhibitors) compared to that of the control (without inhibitors).

All three inhibitors have strong activity against GLA and can prevent the activation of E.MMP.A vectors by exogenous GLA (Table 5). However, unlike TIMP-2 and CT 1339, TIMP-1 could not efficiently block the activation of E.MMP.A by endogenous MMPs on HT1080 cells (Table 5). An important difference between TIMP-1 and the other inhibitors is that it displays only weak activity against the MT1-MMP expressed on HT1080 cells (Fig. 17. described below). These experiments therefore point to a central role for the MT-MMP in HT1080-mediated activation of the E.MMP.A vector.

Figure 17 is a gelatin zymogram showing the effect of TIMP-1 or a synthetic MMP-inhibitor, CT 1339 on cellular activation of endogenous pro-GLA on HT 1080 cells. The E.MMP.A viral supernatant was incubated on HT 1080 cells for 6 h at 37°C in the absence of any inhibitors (lane 1), in the presence of 10 μg/ml (lane 2) or 30 μg/ml TIMP-1 (lane 3), and 1 μM (lane 4) or 10 μM (lane 5) CT 1339. At the end of the incubation period, an aliquot of the supernatant was loaded onto 7% SDS-PAGE gel containing 0.5 g/ml denatured type I collagen and electrophoresis was carried out at 4°C for 1 h, after which the gel was incubated twice for 15 min each in 2.5% Triton-X 100 to remove the SDS, washed in water and then incubated overnight at room temperature in 100 mM Tris, 30 mM $CaCl_2$, 0.0015% Brij and 0.001% NaN_3. The gel was then stained in 0.25% Coomassie Brilliant Blue Green (Sigma). The location of gelatinolytic activity on the gelatin zymogram is detectable as a clear band in the background of blue staining.

There have been many variably successful attempts to target retroviral vectors through ligand-receptor interactions (Valsesia-Wittmann *et al.*, 1994 J. Virol. *68*, 4609; Cosset *et al.*, 1995 J. Virol. *69*, 6314; Kasahara *et al.*, 1994 Science *266*, 1373; Matano *et al.*, 1995 J. Gen. Virol. *76*, 3165; Somia *et al.*, 1995 Proc. Natl. Acad. Sci. *92*, 7570). Here we have adopted a two-step targeting strategy that allows us to utilise the specificity of protease-substrate interactions to activate the infectivity of receptor-targeted retroviral vectors. We previously relied on the addition of exogenous Factor Xa protease for vector activation, an approach that might have rather limited applications for *in vivo* gene therapy. Here, we have demonstrated for the first time a retroviral vector whose infectivity can be activated by endogenously produced disease-associated proteases. The

vector is optimally cleaved and activated by membrane-associated MMPs on human tumour cell lines.

The targeting strategy that we have pursued may have interesting parallels with the mechanism of HIV entry in which primary virus attachment to CD4 leads to a conformational rearrangement or proteolytic cleavage in gp120, and secondary virus attachment to one of the recently characterised HIV co-receptors (Feng *et al.*, 1996 Science *272*, 872; Deng *et al.*, 1996 Nature *381*, 661; Handley *et al.*, 1996 J. Virol. *70*, 4451). C-type retroviral vectors with engineered SU glycoproteins could therefore be developed as model systems to probe the entry mechanisms that are employed by naturally occurring viruses, such as HIV.

It is hoped that targeted vectors of the type that we have described in this report will open up new possibilities for gene therapy in MMP-associated diseases, for example in cancer, where elevated MMP production in tumour deposits is required for angiogenesis, invasiveness and metastatic potential and is strongly correlated with poor prognosis (Murray *et al.*, 1996 Nature Med. *2*, 461).

Example 6

Retroviral display of trimeric binding domains, TNF alpha and CD40 ligand.

The following experiments demonstrate that chimaeric envelopes bearing TNF alpha or CD40 ligand as an N-terminal extension can be incorporated into retroviral vector particles where it appears that the trimeric binding domain forms a cap over the envelope glycoprotein to which it is fused. The amphotropic infectivity of the vectors incorporating these chimaeric envelopes is therefore low but is greatly enhanced by cleaving the trimeric ligand from their surface.

Cell Lines

The TELCeB6 cell line has been described in the preceding examples. The NIH 3T3, A431 (human squamous carcinoma; ATCC CRL1555) and HT1080 (human fibrosarcoma; ATCC CCL121) cell lines were grown in DMEM (Gibco-BRL, UK) supplemented with 10% fetal calf serum (FCS; PAA Biologicals, UK), benzylpenicillin (60 mg/ml) and

streptomycin (100 mg/ml) at 37°C in an atmosphere of 5% CO_2. The B cell lines, Daudi (human Burkitt's lymphoma; ATCC CCL 213), Raji (human Burkitt's lymphoma; ATCC CCL 86) and K422 (human Non-Hodgkin B cell; Dryer *et al.*, 1990 Blood *75*:709-714), and T cell line, Jurkat (human acute T cell leukemia; ATCC TIB 152) were grown in RPMI 1640 (Gibco-BRL) supplemented with 10% FCS, benzylpenicillin (60 mg/ml) an streptomycin (100 mg/ml) at 37°C in an atmosphere of 5% CO_2.

Construction of chimaeric envelope expression vectors

The human tumour necrosis factor-alpha (TNF-a)-4070A SU chimaeric envelope expression vectors TNF-a.A, TNF-a.GS.A, TNF-a.X.A, TNF-a.XA, and TNF-a.MMP.A, have an TNF-a cDNA (Wang *et al.*, 1995 Science, *228*: 149-154), flanked by *Sfi*I and *Not*I restriction sites, inserted at codon +1 of the N-terminus of wild type 4070A MLV SU envelope by different linkers (Fig. 18). The TNF-a.A vector is linked via a 3 alanine (AAA) linker; TNF-a.GS.A via a non-cleavable $AAAG_4S$ linker; TNF-a.X.A via Factor Xa protease cleavable linker (AAAIEGR) and TNF-a.MMP.A via an MMP-cleavable linker (AAAPLGLWA) (single letter amino acid code). The Factor Xa protease cleaves IEGR after the arginine residue and the PLGLWA linker is susceptible to gelatinase A (MMP-2) and MT-MMP between the glycine and leucine residues.

The CD40L-4070A SU chimaeric envelope expression vectors have part of the CD40L cDNA, flanked by *Sfi*I and *Not*I restriction sites, inserted at codon +1 of the N-terminus of 4070A MLV by the 4 different linkers as mentioned above. The vectors are termed CD40L.A, CD40L.GS.A, CD40L.X.A and CD40L.MMP.A (Fig. 19).

A PCR derived *Sfi*I-*Not*I DNA fragment encoding the 155 amino acids of the trimeric human TNF-a was generated using a cDNA template and two primers, sTNFback (5'> CCG GTA CCG GCC CAG CCG GCC TCT TCT TCT CGT ACC CCG, Seq ID No. 29) with a *Sfi*I site, and
nTNFfor (5'> AAG TCT TAG CGG CCG CCA GAG CGA TGA TAC CGA AG, Seq ID No. 30) with a *Not*I site.

The *Sfi*I-*Not*I PCR fragment encoding the 145 amino acids of the soluble extracellular

domain of the trimeric CD40L (Gly 116-Leu 261; Karpusas *et al.*, 1995 Structure, *3*: 1031-1039) was generated using a cDNA template (ATCC 79813) and two primers: sCD40Lb (5' > CCG GTA CCG GCC CAG CCG GCC GGT GAT CAG AAT CCT CAA ATT GC, Seq ID No. 31) with a *Sfi*I site and
nCD40Lf (5' > AAG TCT TAG CGG CCG CGA GTT TGA GTA AGC CAA AGG, Seq ID No. 32) with a *Not*I site. The respective PCR fragments were digested with *Sfi*I and *Not*I restriction enzymes and cloned into the *Sfi*I-*Not*I digested EA.1 backbone or EXA.1 to obtain TNF-a.A or CD40L.A. and TNF-a.X.A or CD40L.X.A, respectively (Nilson *et al.*, 1996 Gene Therapy *3*: 280-286).

To obtain TNF-a.GS.A or CD40L.GS.A, and TNF-a.MMP.A or CD40L.MMP.A, the respective *Sfi*I-*Not*I digested TNF-a or CD40L PCR fragments were cloned into *Sfi*I-*Not*I digested E.GS.A or E.MMP.A backbones, respectively (Peng *et al.*, A gene delivery system activatable by disease-associated matrix metalloproteinases, submitted). The sequences of the constructs were checked and verified by DNA sequencing.

Production of viruses

The various TNF-a and CD40L envelope expression plasmids were stably transfected by calcium phosphate precipitation (Sambrook *et al.*, 1989, Molecular cloning: A laboratory manual) into the TELCeB6 packaging cells. Transfected cells, grown in 10% FCS-DMEM at 37°C, were selected with 50 μg/ml phleomycin (Sigma, Poole, Dorset, UK). Resistant colonies were pooled and expanded, and before harvest, the confluent cells were tranferred to 32°C for 72 h. The viral supernatants were then harvested and filtered (0.45 μm, Acrodisc, Gelman Sciences MI, USA) after overnight incubation of the confluent cells with serum free DMEM at 32°C. These filtered supernatants were then used either for immunoblotting, binding or infection assays.

Immunoblots

For immunoblotting, the viral particles were pelleted by ultracentrifugation of the flitered viral supernatant (Beckman, USA) at 30,000 rpm for 1 h at 4°C in a SW 40 rotor. The pellet was then resuspended in 100 μl cold PBS and stored at -70°C till further analysis. An aliquot (10 μl) of the viral proteins was separated by electrophoresis on a 10% SDS-

PAGE gel, electrotransferred onto nitrocellulose membrane (Hybond ECL, Amersham Life Sciences, UK) and detected by immunostaining with goat antisera raised against Rausher leukemia virus gp 70-SU envelope protein (Quality Biotech, USA), followed by horseradish peroxidase-conjugated rabbit anti-goat immunoglobulins antibodies (DAKO, Denmark) and developed with an enhanced chemiluminescence kit (Amersham).

To detect the presence of processed (SU) and unprocessed (SU + TM) in the cells, the viral complementing cells were grown to confluency on petri dishes (10 cm in diameter), washed once in cold PBS and then incubated for 10 min at 4°C with cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton-X, 0.05% sodium dodecyl sulfate, 5 mg/ml sodium doxycholate and 1 mM PMSF. The lysed cells were scraped from the plates and the suspension centrifuged at 10,000 xg for 20 min to pellet the nuclei. Thirty μl of the supernatant was used for electrophoresis and immunoblotting.

Results:

There were no visible bands of envelope proteins seen on the immunoblots for chimaeric TNF-a-4070A SU indicating little or no envelope expression. When the cell lysates were tested, there was unprocessed (SU + TM) chimaeric envelope proteins but little detectable processed (SU) envelope proteins. Results from the immunoblots for CD40L-4070A SU indicated that there was chimaeric envelope expression and incorporation, and expression between the chimaeric envelopes were comparable except for CD40L.MMP.A-SU which is most highly expressed.

Infection Assays

i. Infection of cells by chimaeric vectors-blocking infectivity of 4070A by trimeric ligand

The TNF-a and CD40L chimaeric vectors were tested for infectivity on NIH 3T3, A431 and HT1080 cells. The cells were seeded overnight at 37°C at a density of approximately 1 x 10^5 cells per individual well in a 6-well tissue culture plate (Corning, New York). The medium was removed the next day and cells were washed once in serum-free DMEM. An aliqout (1 ml) of the filtered viral supernatant was used to infect the cells in the presence of 8 μg/ml polybrene. At the end of the 6 h incubation period, the medium was

removed and the cells washed once in serum-free DMEM and 10% FCS-DMEM was added. The cells were then incubated for 72 h at 37°C before they were stained with X-gal. The cells were washed once in cold PBS, fixed in 0.5% glutaldehyde-PBS for 15 min, washed once in cold PBS and incubated with X-gal overnight at 37°C. Number of colonies transduced with the β-galactosidase gene (blue colonies) were counted and the titre expressed as enzyme forming units (efu)/ml viral supernatant.

ii. Treatment of viral supernatants with Factor Xa protease: reversal of blockage to infectivity by protease

An aliquot (1 ml) of the filtered supernatant was incubated with 2.5 mM $CaCl_2$ in the presence or absence of 4 μg/ml Factor Xa protease (New England Biolabs, UK) for 1 h at 37°C. At the end of the incubation period, the supernatant was added onto the NIH 3T3 or HT 1080 cells for 6 h before the supernatant was removed, cells washed and maintained in 10% FCS-DMEM before they were stained with X-gal as before.

Results:

TNF-a-4070A chimaeras

The titre of the TNF-a-4070A vectors on NIH3T3 and HT1080 cells were low (Table 6). This low level of infectivity could be due to the low level of chimaeric envelope expression. However, it could also be due to the display of the trimeric TNF-a on the 4070A-SU. The trimer was able to block the infectivity of the amphotropic vector, which would be otherwise be highly infective on the murine NIH 3T3 cells, which do not bear the human TNF-a receptor.

Table 6. Titre of TNF-a-4070A vectors on cell lines in presence of 8 μg/ml polybrene

efu/ml	TNF-a.A	TNF-a.G_4S.A	TNF-a.X.A	TNF-a.MMP.A	4070A
NIH3T3	0	7	5	41	1×10^7
HT1080	0	0	0	11	1×10^7

This blockage of infectivity by the trimeric TNF-a could be reversed by the addition of factor Xa protease to cleave off the TNF-a ligand on the TNF-a.X.A vector and thus,

allowing the 4070A-SU to bind and infect the cells. As a result, the titre of the TNF-a.X.A vector increased 4-fold and 60-fold, respectively, on HT1080 cells and NIH 3T3 cells after treatment with Factor Xa protease (Table 7).

Table 7. Titre (efu/ml) of TNF-a.X.A on cells in absence or presence of Factor Xa protease

	NIH 3T3	HT1080
-FXa	10	32
+Fxa	595	136

CD40L-4070A chimaeras

The infectivity of the CD40L-4070A chimaeras are significantly lower than that of the wild type on NIH 3T3, A431 and HT1080 cells (Table 8), indicating that the display of CD40L on the envelope is blocking the infectivity of the vector.

Table 8. Titre (efu/ml) of CD40L-4070A vectors on cell lines in presence of 8 μg/ml polybrene

efu/ml	CD40L.A	CD40L.G_4S.A	CD40L.X.A	CD40L.MMP.A	4070A
NIH3T3	6.6×10^3	1.6×10^4	2.9×10^3	2.2×10^4	1×10^8
A431	122	2.3×10^3	not done	1.4×10^3	1×10^8
HT1080	4.6×10^2	8.2×10^3	3.4×10^2	1.9×10^4	1×10^8

Upon treatment of the CD40L.X.A with Factor xa protease, the infectivity of the vector is increased dramatically (Table 9).

Table 9. Titre (efu/ml) of CD40L.X.A in absence or presence of Factor-Xa protease

	NIH 3T3	A431	HT1080
-Fxa	2.9×10^3	116	97
+Fxa	1×10^6	1×10^6	1×10^6

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Medical Research Council
(B) STREET: 20 Park Crescent
(C) CITY: London
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(F) POSTAL CODE (ZIP): W1N 4AL
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(ii) TITLE OF INVENTION: Recombinant Viruses Incorporating a Protease Cleavable Protein

(iii) NUMBER OF SEQUENCES: 32

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCAAATCTGC GGCCGCAATC GAGGGAAGGC CTCATCAAGT CTATAATATC ACC 53

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCAAATCTGC GGCCGCAGCT TCGCCCGGCT CCAGTCC 37

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCAAATCTGC GGCCGCAATC GAGGGAAGGG CTTCGCCCGG CTCCAGTCC 49

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCAAATCTGC GGCCGCAATG GCAGAGAGCC CCCATC 36

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCAAATCTGC GGCCGCAATC GAGGGAAGGA TGGCAGAGAG CCCCCATC 48

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCCAGAACGG GGTTTGGCC 19

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CTGCAAGCCC ACATTGTTCC 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ala Ala Ala Gly Gly Gly Gly Ser Ile Glu Gly Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Ala Ala Ala Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly
1 5 10 15

Gly Ser Ile Glu Gly Arg
20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCAAATCTGC GGCCGCAGGT GGAGGCGGTT CAATCGAGGG AAGGATGGCA GAG 53

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 83 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCAAATCTGC GGCCGCAGGT GGAGGCGGTT CAGGCGGAGG TGGCTCTGGC GGTGGCGGAT 60

CGATCGAGGG AAGAATGGCA GAG 83

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGCATTCATG CGGCCGCGGC CCAGCCGGCC ATGAAGCAAC TAGAAGACAA GGTGGAGGAA 60

CTCCTTAGCA AGGTATACC 79

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GCAAATCTGC GGCCGCCTCT CCAACAAGCT TCTTCAGTCG AGCGACTTCG TTCTCAAGAT 60

GGTATACCTT GCTAAGGAG 79

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGCATTCATG CGGCCGCGGC CCAGCCGGCC ATGAAGCAAG CAGAAGACAA GGCAGAGGAA 60

GCTCTTAGCA AGGCTTACC 79

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GCAAATCTGC GGCCGCCTCT CCAGCAAGCT TCTTTGCTCG AGCAGCTTCG TTCTCTGCAT 60

GGTAAGCCTT GCTAAGAGC 79

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGCATTCATG CGGCCGCGGC CCAGCCGGCC ATGAAGCAAA TCGAAGACAA GATAGAGGAA 60

ATTCTTAGCA AGATCTACC 79

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 82 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GCAAATCTGC GGCCGCCTCT CCTATAAGCT TCTTGATTCG AGCAATTTCG TTCTCTATAT 60

GGTAGATCTT GCTAAGAATT TC 82

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGCATTCATG CGGCCGCGGC 20

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GCAAATCTGC GGCCGCCTCT C 21

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Ala Ala Ala Ile Glu Gly Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Ser Ala Ala Ile Glu Gly Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GACAAGCTAG AGGAAGTACT TAGCAAGCTC TACCATGTCG AGAACGAACT TGCTCGAGTT 60

AAGAAG 66

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGCATTCATG CGGCCGCAAT GAAGCAAGTG GAAGACAAGC TAGAGGAAGT AC 52

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GCAAATCTGC GGCCGACTCT CCCAGAAGCT TCTTAACTCG AGCAAGTTC 49

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Ala Ala Ala Gly Gly Gly Gly Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Ala Ala Ala Pro Leu Gly Leu Trp Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GCAAATCTGC GGCCGCACCT TTGGGACTTT GGGCAATGGC AGAGAGCCCC CATC 54

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GCAAATCTGC GGCCGCAGGT GGAGGCGGTT CAATGGCAGA GAGCCCCCAT C 51

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CCGGTACCGG CCCAGCCGGC CTCTTCTTCT CGTACCCCG 39

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

AAGTCTTAGC GGCCGCCAGA GCGATGATAC CGAAG 35

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CCGGTACCGG CCCAGCCGGC CGGTGATCAG AATCCTCAAA TTGC 44

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

AAGTCTTAGC GGCCGCGAGT TTGAGTAAGC CAAAGG 36

Claims

1. A recombinant viral particle capable of infecting a eukaryotic cell, the viral particle comprising: a substantially intact viral glycoprotein fused, via an intervening linker region, to a heterologous polypeptide displayed on the surface of the particle, which heterologous polypeptide modulates the ability of the viral particle to infect one or more eukaryotic cell types and is cleavable from the viral glycoprotein by a protease acting selectively on a specific protease cleavage site present in the linker region, such that cleavage of the heterologous polypeptide from the viral glycoprotein allows the glycoprotein to interact normally with its cognate receptor on the surface of a target cell.

2. A particle according to claim 1, further comprising the nucleic acid sequence encoding the protease cleavage signal.

3. A particle according to claim 1 or 2, wherein the heterologous polypeptide has specific binding affinity for a cognate receptor on the surface of an eukaryotic cell, binding to which does not allow infection of the cell by the viral particle.

4. A particle according to claim 1 or 2, wherein the heterologous polypeptide has no specific binding affinity for a eukaryotic cell surface component.

5. A particle according to any one of the preceding claims, wherein the heterologous polypeptide sterically hinders binding of the viral glycoprotein to its cognate receptor on the eukaryotic cell.

6. A particle according to any one of the preceding claims, wherein heterologous polypeptide sterically hinders fusion of an enveloped viral particle with an eukaryotic cell to which it is bound.

7. A particle according to any one of the preceding claims, wherein the heterologous polypeptide is displayed as an oligomer.

8. A particle according to claim 7, wherein the heterologous polypeptide is displayed as a dimer or trimer.

9. A particle according to claim 7 or 8, wherein the heterologous polypeptide undergoes oligomerisation with the same stoichiometry as that with which the fused viral glycoprotein oligomerises.

10. A particle according to any one of the preceding claims, wherein the protease cleavage site is accessible to the relevant protease (i.e. that which recognises the cleavage site) before the viral particle becomes bound to an eukaryotic cell.

11. A particle according to any one of claims 1 to 9, wherein the protease cleavage site becomes accessible to the relevant protease only after the viral particle has become bound to an eukaryotic cell.

12. A particle acording to claim 11, wherein the protease cleavage site becomes accessible after the heterologous polypeptide has bound to its cognate receptor on the eukaryotic cell.

13. A particle according to claim 11 or 12, wherein the protease cleavage site becomes accessible after the viral glycoprotein has bound to its cognate receptor on the eukaryotic cell.

14. A particle according to any one of the preceding claims, wherein the protease cleavage site is cleaved by a protease selected from the group consisting of: serine proteases; cysteine proteases; aspartic proteases; matrix metalloproteinases (MMP); and membrane-associated proteases.

15. A particle according to claim 14, wherein the protease cleavage site is cleaved by a protease selected from the group consisting of: factor Xa; gelatinase A; membrane-type MMP (MT-MMP); urokinase; streptokinase; tissue plasminogen activator (tPA); and plasmin.

16. A particle according to any one of the preceding claims, wherein the protease cleavage site is cleaved by a protease involved in one or more of the following processes: tissue remodelling; wound healing; inflammation; and tumour invasion.

17. A particle according to any one of the preceding claims, suitable for targeted delivery of a nucleic acid to a specific eukaryotic target cell.

18. A particle according to any one of the preceding claims, comprising an adenovirus.

19. A particle according to any one of claims 1 to 17, comprising an enveloped virus.

20. A particle according to claim 19, comprising a retrovirus.

21. A particle according to claim 19 or 20, comprising a C-type retrovirus.

22. A nucleic acid construct, comprising a sequence encoding a fusion protein, the fusion protein comprising a substantially intact viral glycoprotein fused, via an intervening linker region, to a heterologous polypeptide, wherein the fusion protein is capable of being incorporated into a viral particle capable of infecting an eukaryotic cell, and further wherein the heterologous polypeptide modulates the ability of the viral particle to infect one or more eukaryotic cell types, but cleavage of the heterologous polypeptide from the fusion protein allows the viral glycoprotein to interact normally with its cognate receptor on the surface of the eukaryotic cell.

23. A library comprising a plurality of nucleic acid constructs according to claim 22, wherein at least part of the sequence encoding the intervening linker region is randomised in each construct, such that each construct comprises one of a plurality of different linker regions which are represented in the library.

24. A library of viral particles in accordance with any one of claims 2 to 21, each particle comprising a single nucleic acid construct from a library in accordance with claim 23.

25. A method of screening nucleic acid sequences for those which encode an amino acid sequence which may or may not be cleaved by a protease present in the export pathway of an eukaryotic cell, comprising: causing the expression of a plurality of nucleic acid sequences in eukaryotic cells, each sequence encoding a substantially intact viral glycoprotein fused to a heterologous polypeptide via a randomised intervening linker region, the presence of the heterologous polypeptide serving to inhibit the interaction of the viral glycoprotein with its cognate receptor, and wherein each nucleic acid sequence further comprises a packaging signal allowing for viral incorporation, such that those intervening linkers which are recognised by a protease present in the export pathway of the eukaryotic cells will allow for cleavage of the heterologous polypeptide from the viral glycoprotein, resulting in the production of an infectious viral particle; and recovering those nucleic acid sequences directing the expression of such cleavable linker regions from an infected cell.

26. A method of screening nucleic acid sequences for those which encode an amino acid sequence which may or may not be cleaved by a protease, comprising: causing the expression of a plurality of nucleic acid sequences in eukaryotic cells, each sequence encoding a substantially intact viral glycoprotein fused to a heterologous polypeptide via a randomised intervening linker region, the presence of the heterologous polypeptide serving to inhibit the fusion of a viral particle with a eukaryotic cell to which it is bound, and wherein each nucleic acid sequence further comprises a packaging signal allowing for viral incorporation; enriching the viral particles so produced for those which retain the heterologous polypeptide (and so are non-infectious); and contacting the enriched particles with a susceptible eukaryotic cell comprising, or in the presence of, a protease such that those intervening linkers which are recognised by the protease will allow for cleavage of the heterologous polypeptide from the viral glycoprotein, resulting in productive infection of the eukaryotic cell; and recovering those nucleic acid sequences directing the expression of such cleavable linker regions from the infected cell.

27. A method according to claim 26, wherein the protease is exogenously added or is present in the import pathway of the susceptible eukaryotic cell.

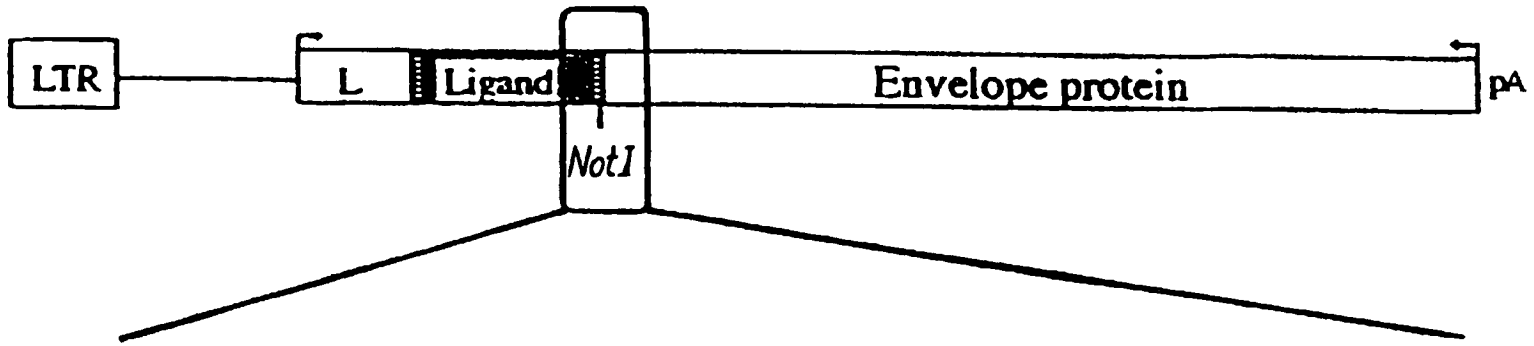
28. A kit for performing a method according to any one of claims 25, 26 or 27 comprising: a nucleic acid construct which comprises a packaging signal allowing for viral incorporation, and a sequence encoding a fusion protein comprising a substantially intact viral glycoprotein, a randomised intervening linker region or a portion of DNA capable of receiving such a randomised sequence, and a heterologous polypeptide which serves to inhibit an interaction of the viral glycoprotein with its cognate receptor on a eukaryotic cell; and instructions for use.

29. A kit according to claim 28, further comprising a eukaryotic cell capable of infection by a virus comprising the substantially intact viral glycoprotein.

30. A method of selectively delivering a nucleic acid to a target eukaryotic cell present among non-target cells, comprising administering to the target and non-target cells a recombinant viral particle capable of infecting eukaryotic cells, the particle comprising: the nucleic acid to be delivered, and a fusion protein comprising a substantially intact viral glycoprotein fused, via an intervening linker region, to a heterologous polypeptide displayed on the surface of the particle, which heterologous polypeptide modulates the ability of the particle to infect one or more eukaryotic cell types and being cleavable from the glycoprotein by a protease acting selectively on a specific protease cleavage site present in the linker region, such that cleavage of the heterologous polypeptide from the glycoprotein occurs preferentially at, or in the vicinity of, the target cell and allows the viral glycoprotein to interact normally with its cognate receptor on the surface of the target cell.

31. A method according to claim 30, wherein the relevant protease is administered exogenously *in vivo*, after administration of the recombinant viral particle.

32. A method according to claim 30, wherein the specific protease is secreted by, or in the same tissue as, the target cells.



	Displayed ligand	*Not I* restriction site	FXa cleavage site	Envelope protein
EMo7		... GAACTGCGC ... E L R EGF	GCGGCCGCA A A A	CCTCATCAA ... P H Q ... Moloney 7-632
EXMo7		... GAACTGCGC ... E L R EGF	GCGGCCGCA A A A	ATCGAGGGAAGG I E G R	CCTCATCAA ... P H Q ... Moloney 7-632
EMo1		... GAACTGCGC ... E L R EGF	GCGGCCGCA A A A	GCTTCGCCC ... A S P ... Moloney 1-632

Fig 1 SHEET 2

Fig. 1 SHEET 1

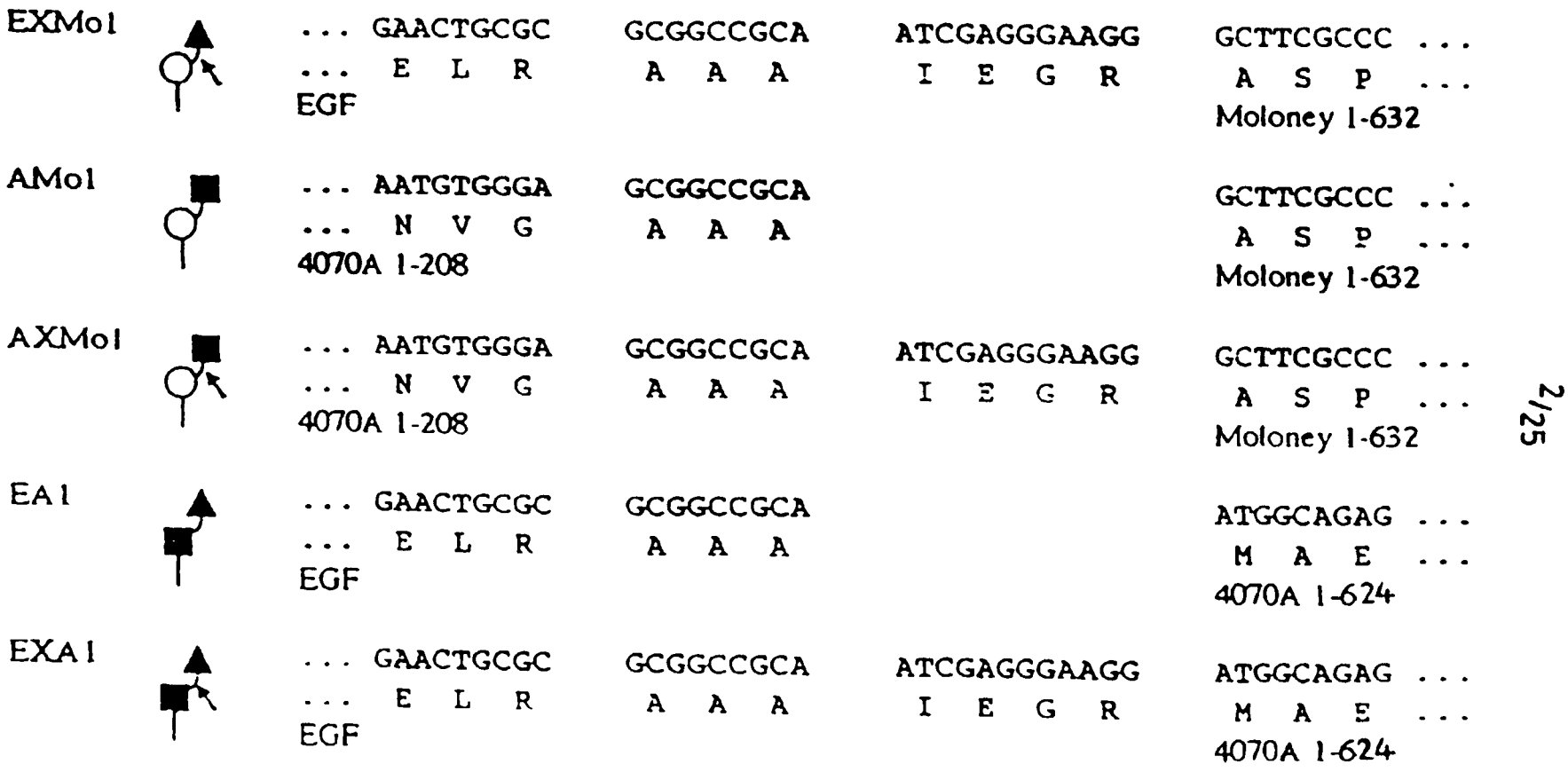


Fig. 1 SHEET 2

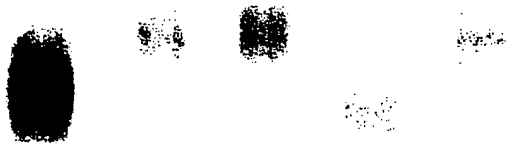


Fig. 2

A B C B C

- factor Xa + factor Xa

Fig. 3

A B C A B C

- factor Xa + factor Xa

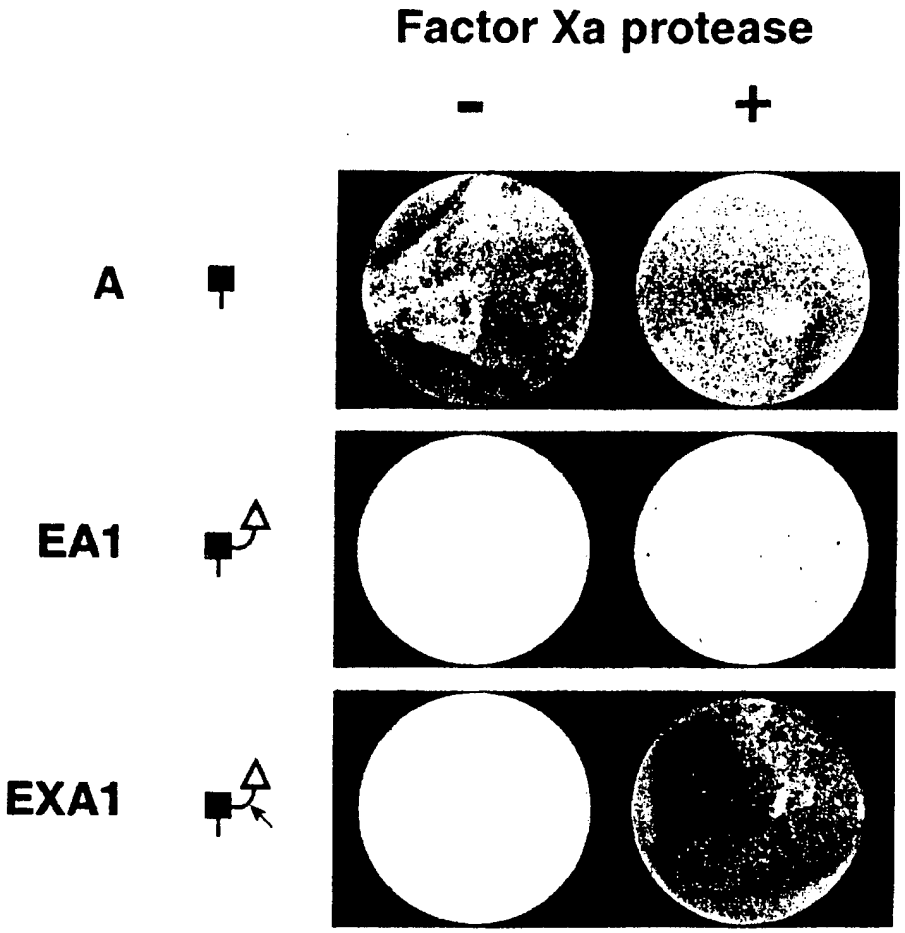


Fig. 4

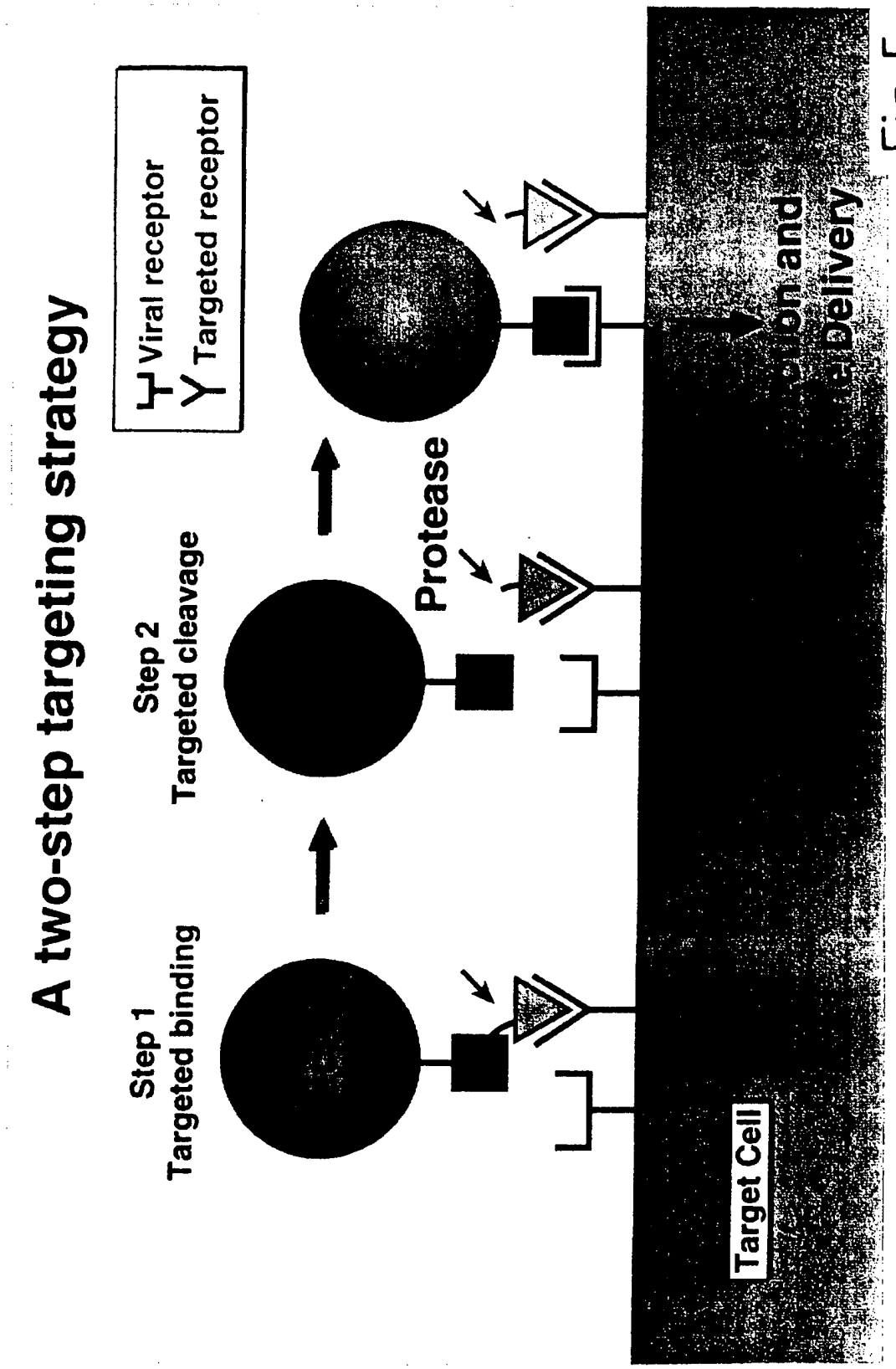

A two-step targeting strategy
Viral receptor
Targeted receptor
Step 1
Targeted binding
Step 2
Targeted cleavage
Protease
Target Cell

Fig. 5

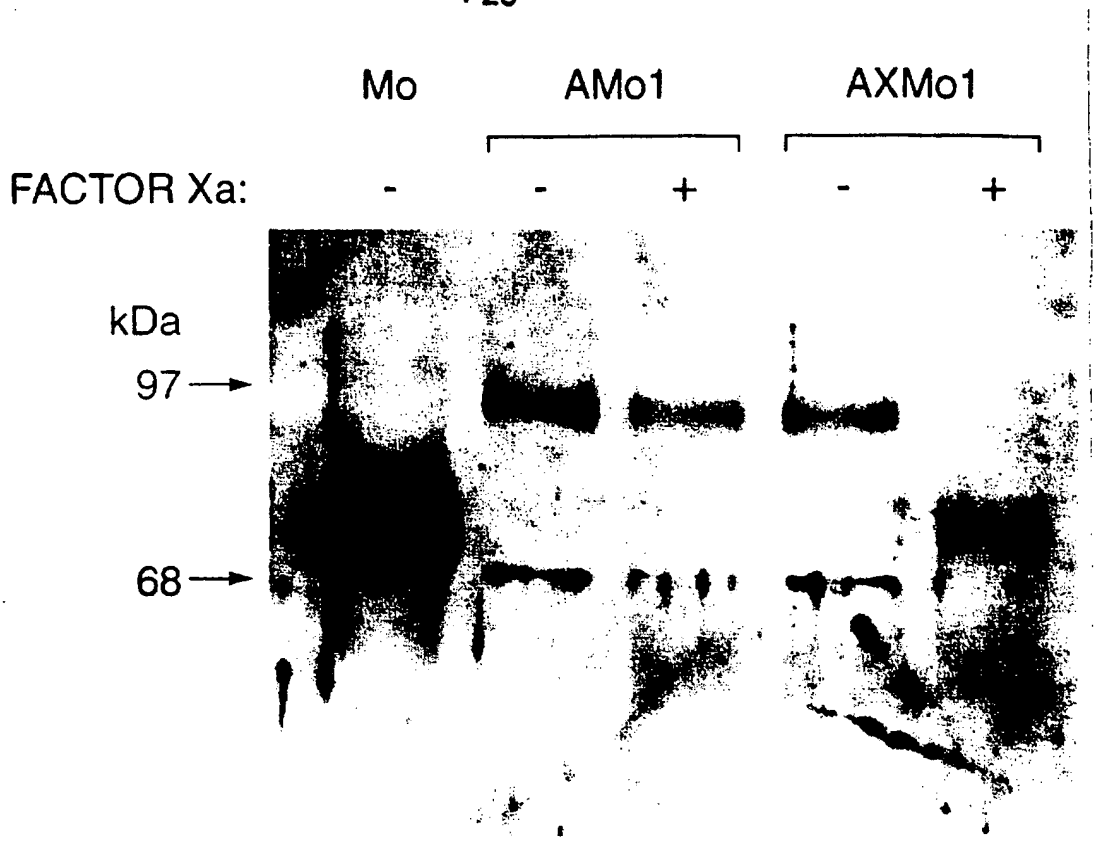

Mo
AMo1
AXMo1
FACTOR Xa:
-
-
+
-
+
kDa
97
68

Fig. 6A

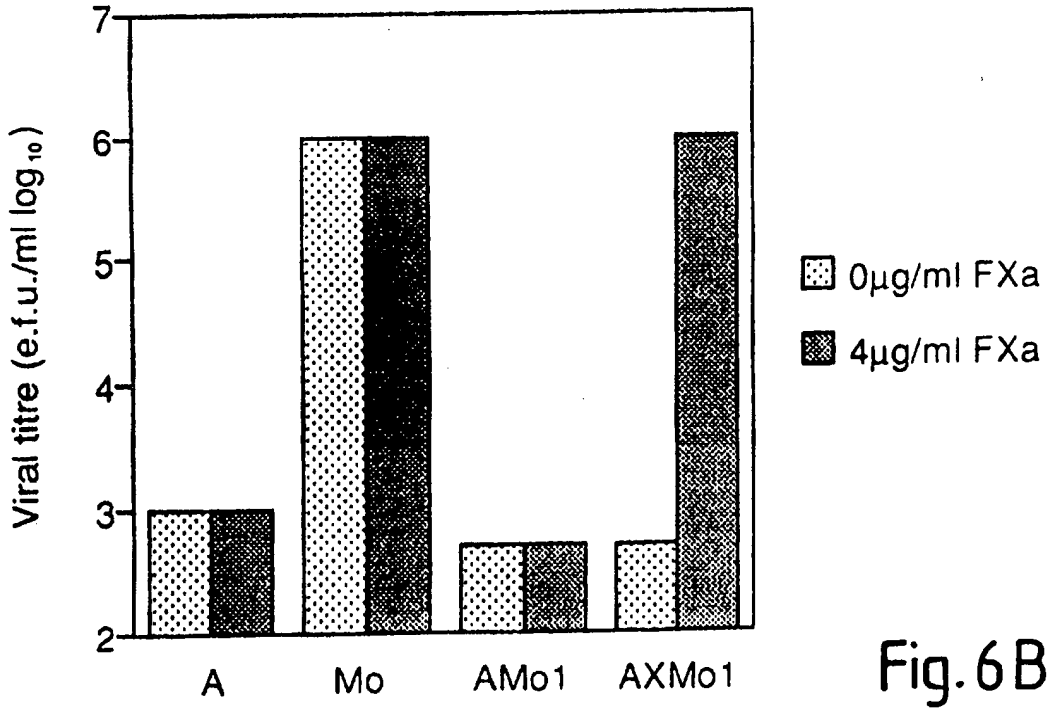
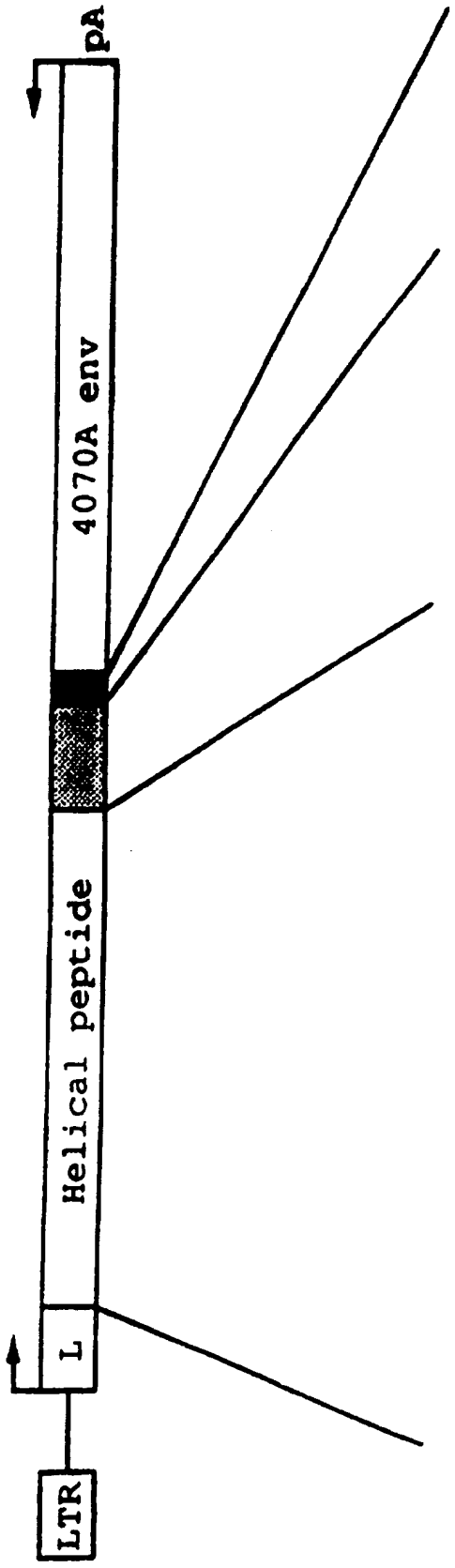

Viral titre (e.f.u./ml log10)
7
6
5
4
3
2
A
Mo
AMo1
AXMo1
0µg/ml FXa
4µg/ml FXa

Fig. 6B



	Linker	FXa site
pAAXA1	AAQPAMKQAEDKAEEALSKAYHAENEAARAKKLAGE	AAA IEGR
pAAGS3XA1	AAQPAMKQAEDKAEEALSKAYHAENEAARAKKLAGE	AAAGGGGSGGGGSGGGGS IEGR

Fig 7 SHEET 2

Fig. 7 SHEET 1

pVLXA1	AAQPAMKQLEDKVEELLSKVYHLENEVARLKKLVGE	AAA	IEGR
pVLGS1XA1	AAQPAMKQLEDKVEELLSKVYHLENEVARLKKLVGE	AAAGGGGS	IEGR
pVLGS3XA1	AAQPAMKQLEDKVEELLSKVYHLENEVARLKKLVGE	AAAGGGGSGGGGSGGGGS	IEGR
pIIXA1	AAQPAMKQIEDKIEEILSKIYHIENEIARIKKLIGE	AAA	IEGR
pIIGS1XA1	AAQPAMKQIEDKIEEILSKIYHIENEIARIKKLIGE	AAAGGGGS	IEGR
pIIGS3XA1	AAQPAMKQIEDKIEEILSKIYHIENEIARIKKLIGE	AAAGGGGSGGGGSGGGGS	IEGR

Fig. 7 SHEET 2

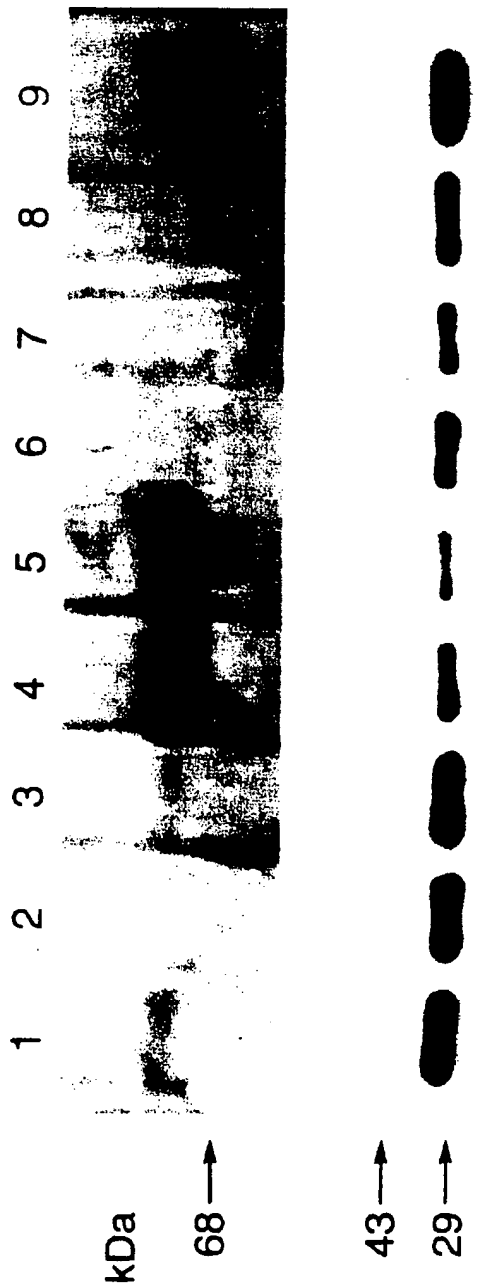


Fig. 8A

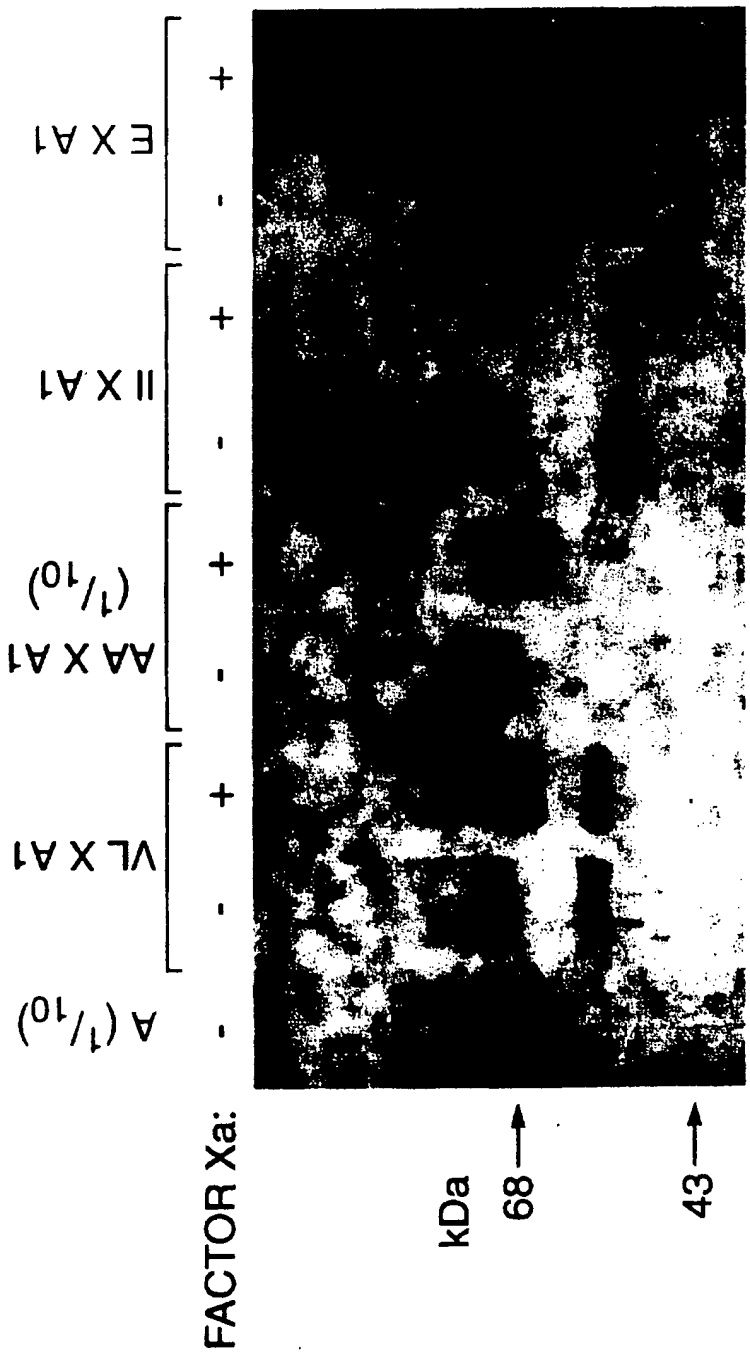


Fig. 8B

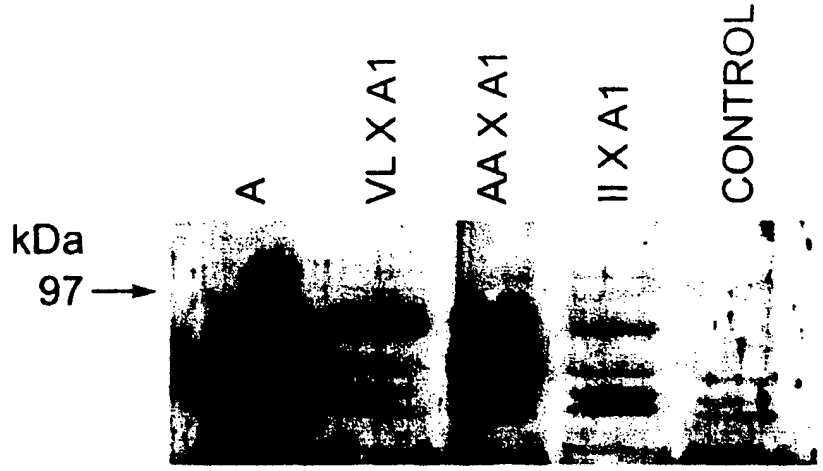


Fig. 8C

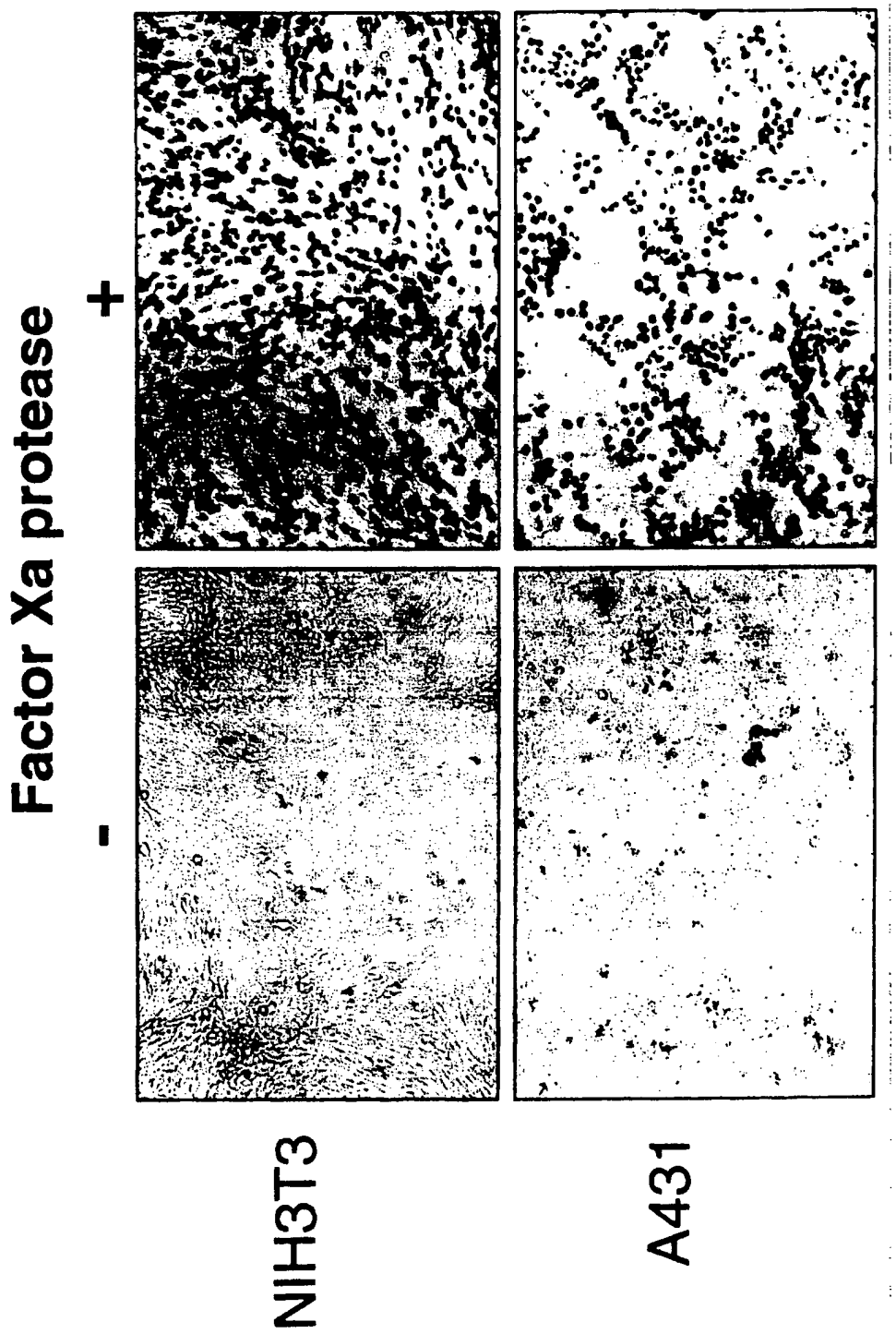
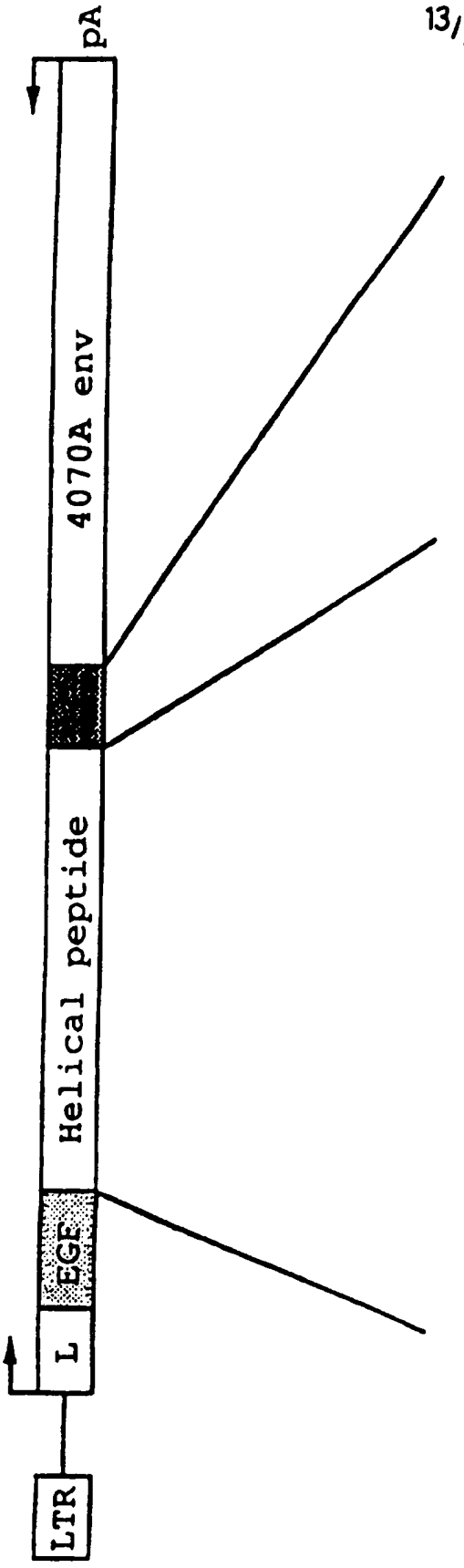


Fig. 9



Linker

pEGF LVA1 AAAMKQVEDKLEEVLSKLYHVENELARVKKLLGE

pEGF LVXA1 AAAMKQVEDKLEEVLSKLYHVENELARVKKLLGE

SAA

SAA IEGR

Factor Xa cleavage site

Fig. 10

DIAGRAMATIC REPRESENTATION OF PLASMID CONSTRUCTS

A

Titre (e.f.u./ml) of harvested β-galactosidase-transducing retroviruses

Factor Xa	A		EGF LVA1		EGF LVXA1	
	−	+	−	+	−	+
NIH3T3	10^7	10^7	<1	1	70	10^3

B

Titre (e.f.u./ml) of harvested β-galactosidase-transducing retroviruses

Factor Xa	A		EGF LVA1		EGF LVXA1	
	−	+	−	+	−	+
A431	10^7	10^7	<1	<1	1	213

Fig. 11
SHEET 1

C

	Titre (e.f.u./ml) of harvested β-galactosidase-transducing retroviruses					
	A		EGF LVA1		EGF LVXA1	
Factor Xa	-	+	-	+	-	+
A431	10^7	10^7	<1	<1	40	340

Reversible inhibition of infection by cleavage of chimaeric envelopes expressing a factor Xa-cleavable, N-terminal oligomerising peptide with an EGF binding domain

Fig. 11
SHEET 2

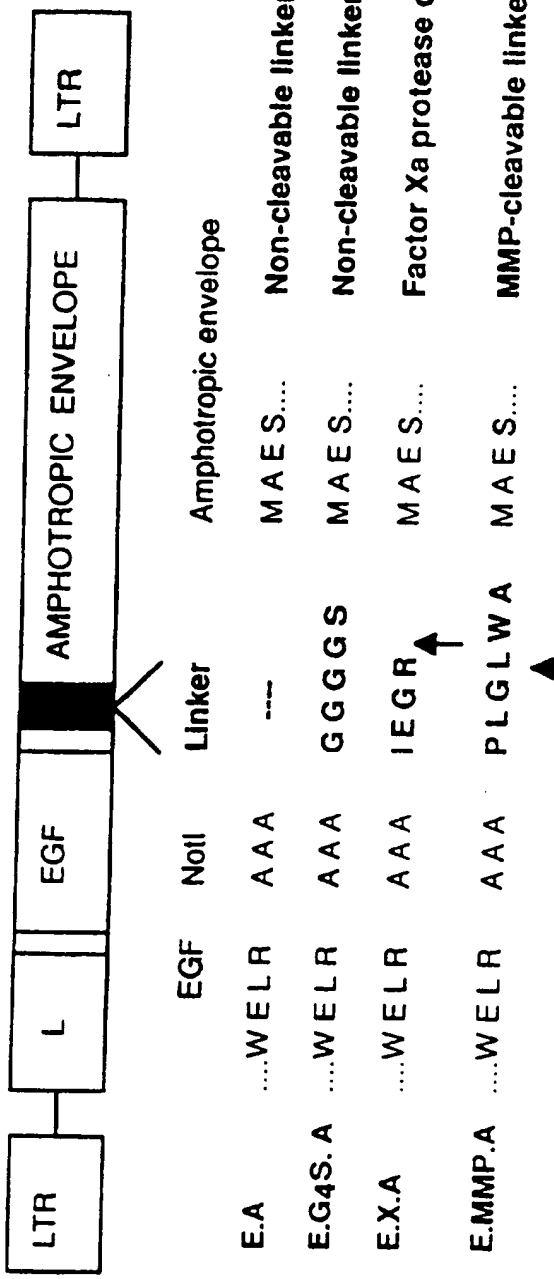

LTR
L
EGF
AMPHOTROPIC ENVELOPE
LTR
SfiI
NotI
EGF NotI Linker Amphotropic envelope
E.A ....WELR AAA --- MAES.... Non-cleavable linker
E.G4S.A ....WELR AAA GGGGS MAES.... Non-cleavable linker
E.X.A ....WELR AAA IEGR MAES.... Factor Xa protease cleavable linker
E.MMP.A ....WELR AAA PLGLWA MAES.... MMP-cleavable linker

Fig. 12

A.

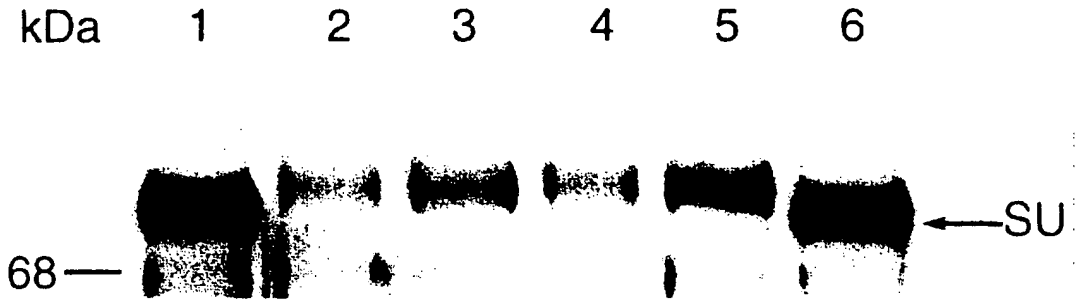


Fig. 13A

B.

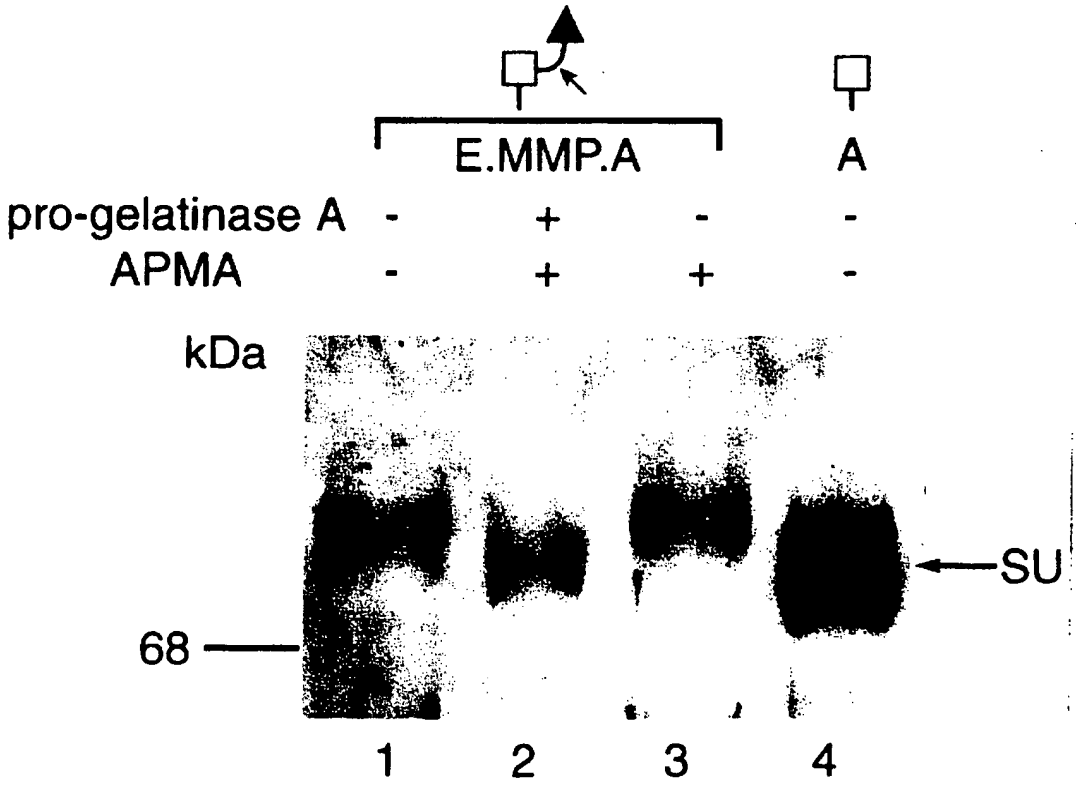


Fig. 13B

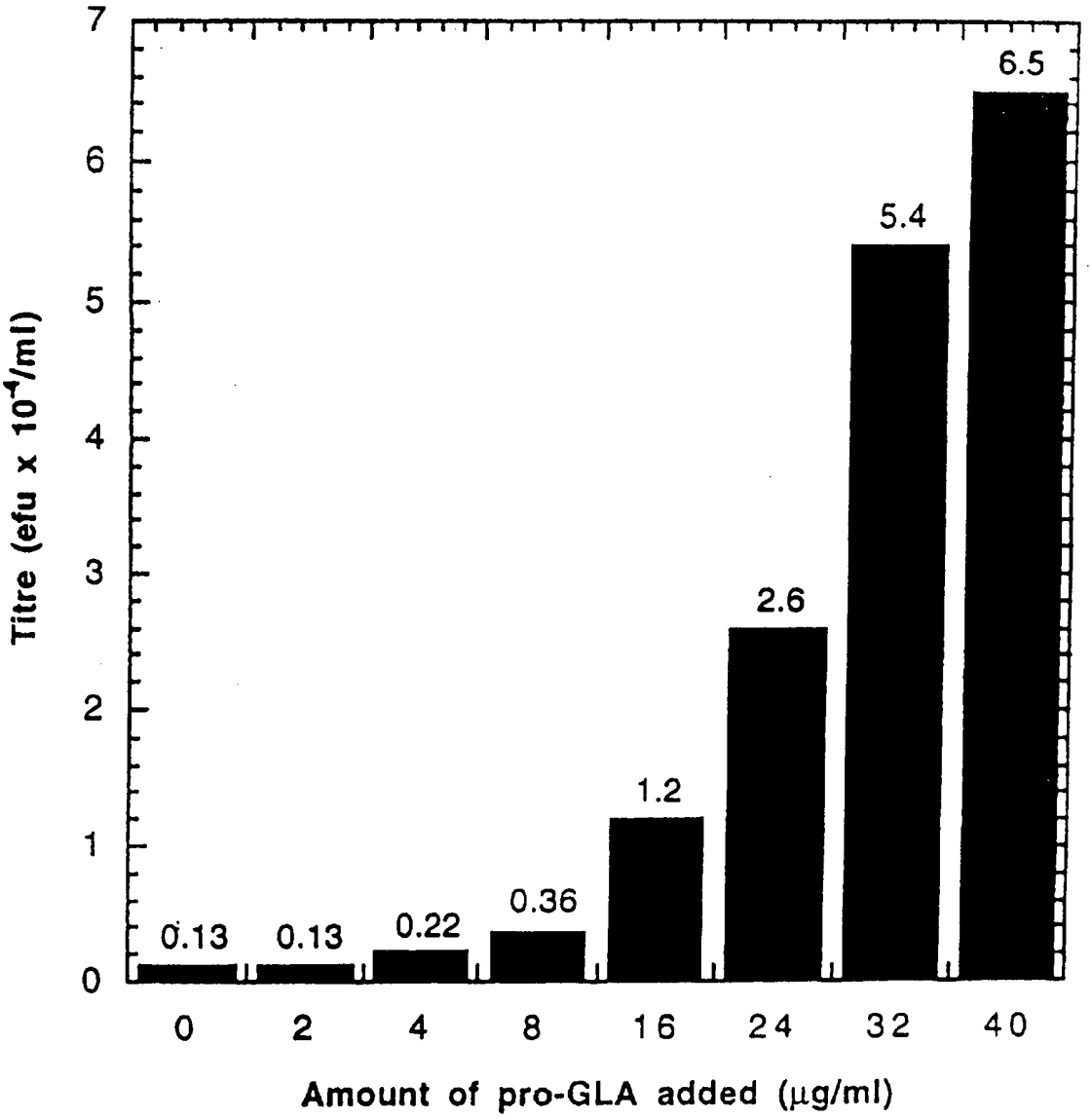

Titre (efu x 10^{-4}/ml)
0
1
2
3
4
5
6
7
0.13
0.13
0.22
0.36
1.2
2.6
5.4
6.5
0
2
4
8
16
24
32
40
Amount of pro-GLA added (µg/ml)

Fig. 14

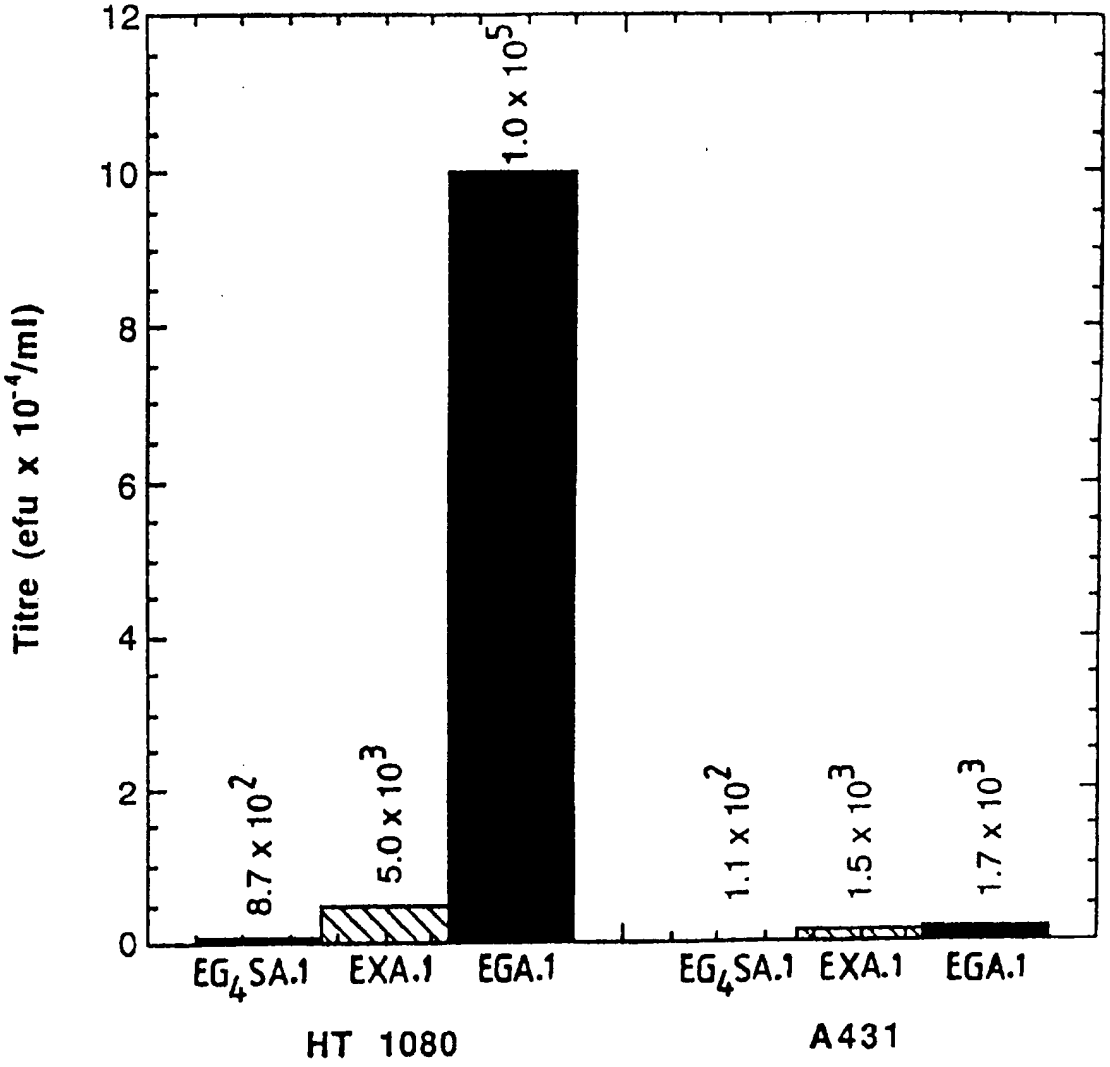
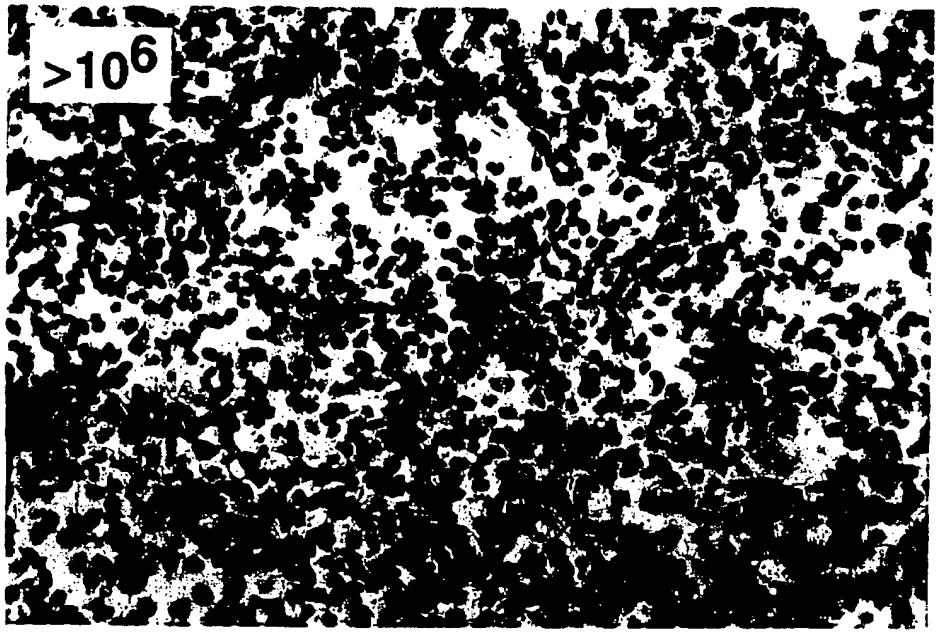
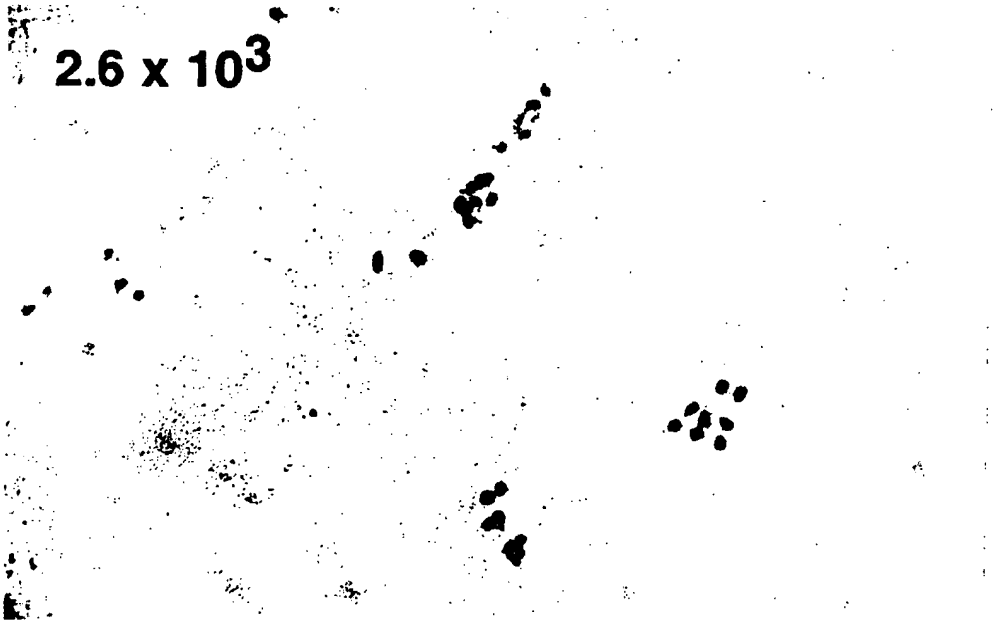

Titre (efu x 10^{-4}/ml)
12
10
8
6
4
2
0
8.7×10^2
5.0×10^3
1.0×10^5
1.1×10^2
1.5×10^3
1.7×10^3
$EG_4SA.1$
EXA.1
EGA.1
$EG_4SA.1$
EXA.1
EGA.1
HT 1080
A431

Fig. 15

E. MMP. A



HT-1080



A431

Fig. 15A

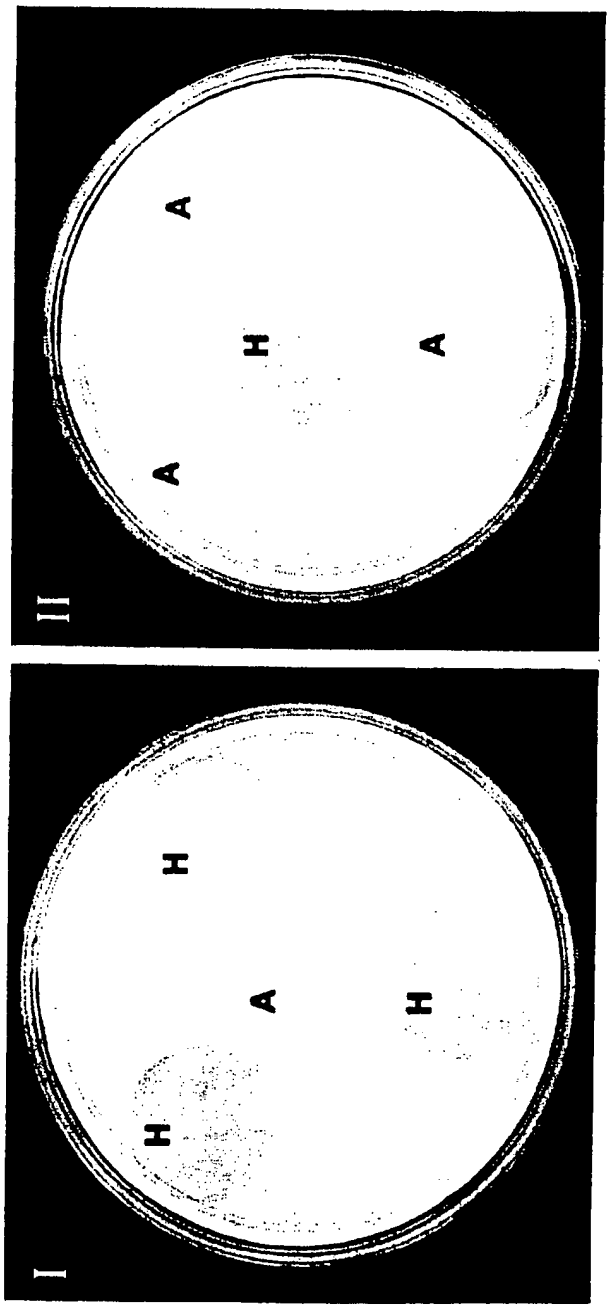


Fig. 16 SHEET 1

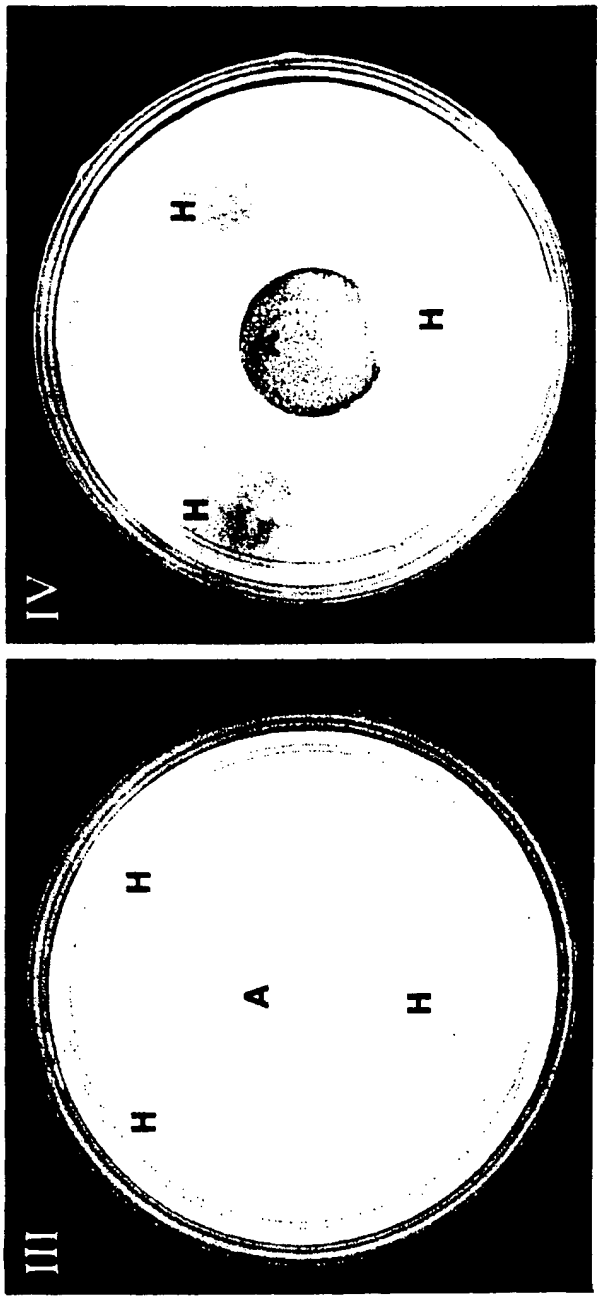


Fig. 16 SHEET 2

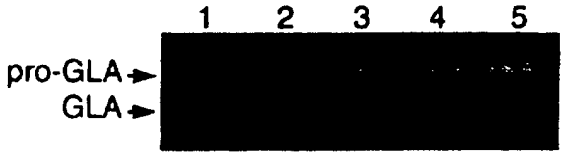


Fig. 17

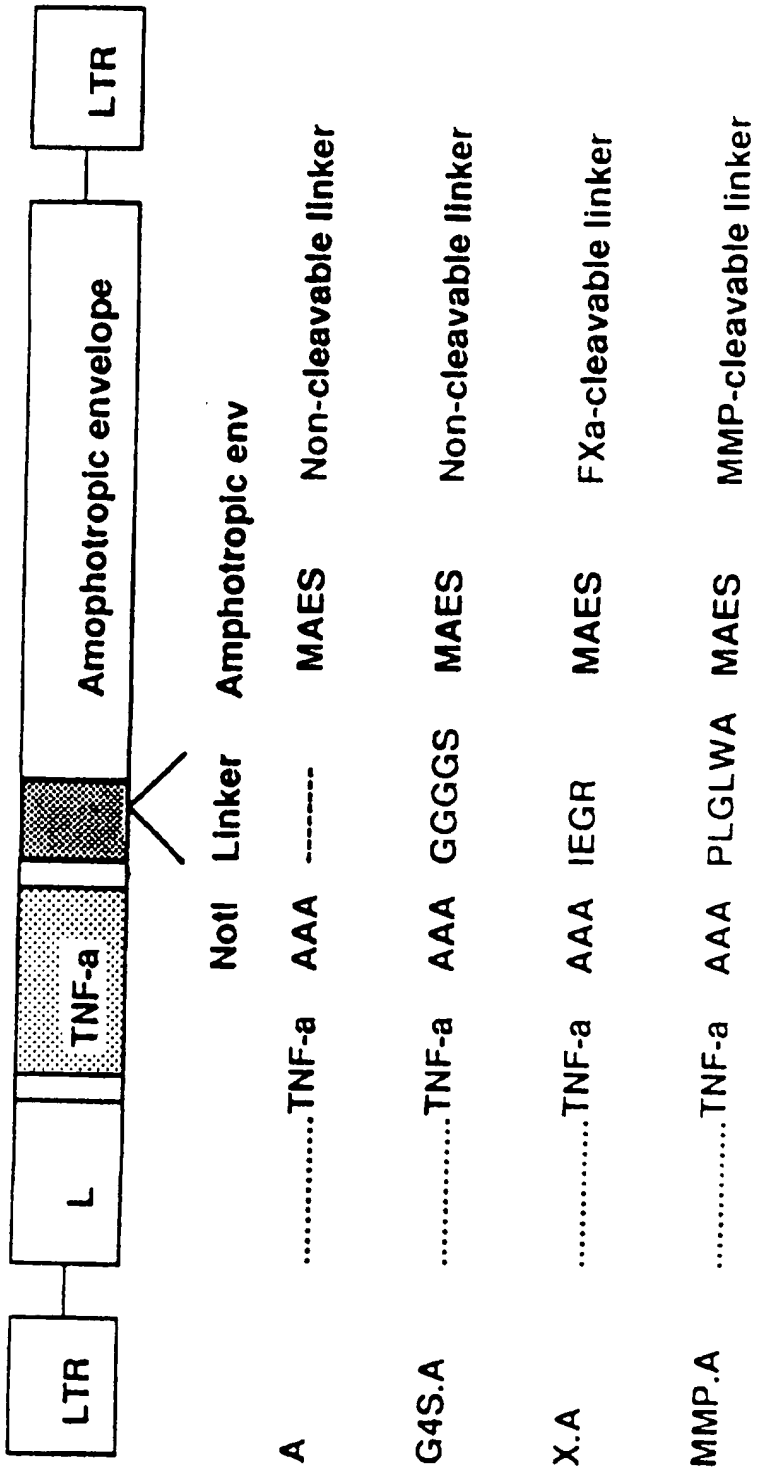
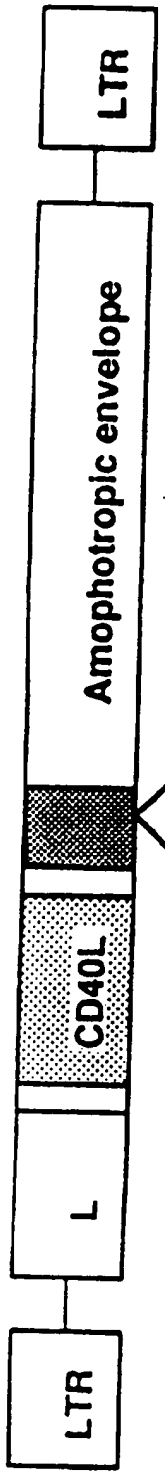

LTR
L
SfiI
TNF-a
NotI
Amophotropic envelope
LTR
NotI Linker Amphotropic env
TNFa.A .....TNF-a AAA ------ MAES Non-cleavable linker
TNFa.G4S.A .....TNF-a AAA GGGGS MAES Non-cleavable linker
TNFa.X.A .....TNF-a AAA IEGR MAES FXa-cleavable linker
TNFa.MMP.A .....TNF-a AAA PLGLWA MAES MMP-cleavable linker

Fig. 18



	NotI	Linker	Amphotropic env	
CD40L.A	.....CD40L	AAA	------	MAES Non-cleavable linker
CD40l.G4S.A	.....CD40L	AAA	GGGGS	MAES Non-cleavable linker
CD40L.X.A	.....CD40L	AAA	IEGR	MAES FXa-cleavable linker
CD40L.MMP.A	.....CD40L	AAA	PLGLWA	MAES MMP-cleavable linker

Fig. 19

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC1/GB 96/02381

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N7/00 C12N15/62 A61K48/00 C07K14/16
C07K14/485 C12Q1/68 C07K14/525 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRITISH JOURNAL OF CANCER, vol. 71, no. sup24, 2 April 1995, page 2 XP002021839 RUSSELL, F. ET AL.: "Retrovirus host range modification by coat protein engineering" see abstract S3 ---	1
A	WO,A,93 11251 (WHITEHEAD BIOMEDICAL INST ;AMERICAN CYANAMID CO (US)) 10 June 1993 see the whole document ---	1,2
A	WO,A,94 06920 (MEDICAL RES COUNCIL ;RUSSELL STEPHEN JAMES (GB); HAWKINS ROBERT ED) 31 March 1994 cited in the application --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
13 January 1997	29.01.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Chambonnet, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,5 179 007 (JARVIS DONALD L ET AL) 12 January 1993 see the whole document	1
A	WO,A,94 27643 (TARGETED GENETICS CORP ;PAUL RALPH W (US); OVERELL ROBERT (US)) 8 December 1994 see claims	1
A	US,A,5 432 018 (DOWER WILLIAM J ET AL) 11 July 1995 see claims	23
P,X	GENE THERAPY, vol. 3, no. 4, April 1996, pages 280-286, XP000609873 NILSON, B.H.K. ET AL.: "Targeting of retroviral vectors through protease-substrate interactions" see the whole document	1
P,X	WO,A,96 26281 (GENVEC INC ;CORNELL RES FOUNDATION INC (US)) 29 August 1996 see claims 1,8-15	1

1

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/GB 96/02381

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 30 and 32 as far as they is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ·onal Application No
PCT/GB 96/02381

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9311251	10-06-93	AU-B-	674134	12-12-96
		AU-A-	3242493	28-06-93
		CA-A-	2123804	10-06-93
		CN-A-	1075334	18-08-93
		CZ-A-	9401273	15-11-95
		EP-A-	0672157	20-09-95
		FI-A-	942623	21-07-94
		HU-A-	67346	28-03-95
		JP-T-	7502403	16-03-95
		NO-A-	942075	03-08-94
		NZ-A-	246276	27-08-96
		ZA-A-	9209418	07-06-93
WO-A-9406920	31-03-94	AU-A-	4827893	12-04-94
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		EP-A-	0670905	13-09-95
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WO-A-9427643	08-12-94	AU-A-	7097494	20-12-94
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		AU-A-	8285291	07-01-92
		EP-A-	0535151	07-04-93
		JP-T-	5508321	25-11-93
		WO-A-	9119818	26-12-91
WO-A-9626281	29-08-96	AU-A-	4980496	11-09-96